JOURNAL OF THE NATIONAL CANCER INSTITUTE

NATIONAL INSTITUTES OF HEALTH • U. S. PUBLIC HEALTH SERVICE

February 1951
Volume 11
Number 4

A SEX DIFFERENCE IN THE KIDNEY GLUCURONIDASE ACTIVITY OF INBRED MICE[1]

ANDREW G. MORROW,[2] DOROTHY M. CARROLL, and EZRA M. GREENSPAN, *Clinical Research Unit, National Cancer Institute, U. S. Marine Hospital, and the Department of Preventive Medicine, The Johns Hopkins University, School of Medicine, Baltimore, Md.*

Investigations of the enzyme glucuronidase were initiated as part of a program of study on the mechanism of action of steroid hormones and chemotherapeutic agents in experimental neoplasia. Results of some of these studies have recently been published (*1*, *2*).

Following the observation by Eschenbrenner (*3*) that chloroform produced renal necrosis in male mice at doses that did not affect female mice, Kerr (*4*) demonstrated a higher level of glucuronidase activity in the kidneys of male mice treated with chloroform as compared to female mice similarly treated. Kerr attributed the elevated glucuronidase activity in males to rapid cell proliferation following injury. No sex difference in the kidney glucuronidase activity of untreated mice was reported.

A sex difference in systemic toxicity, with decreased tolerance in male mice or rats, has been noted for such different substances as carbon tetrachloride (*5*), aminopterin (*6*), and cortisone (*7*). Sex differences in the renal morphology and physiology of mice have been reviewed by Dunn (*8*, *14*).

In the present studies, glucuronidase activity was determined in the kidneys of adult male and female mice of seven inbred strains. The kidneys of immature animals of both sexes were also assayed in three of these strains and compared to the values observed in adult mice. The

[1] Received for publication July 5, 1950.

[2] Present address: Department of Surgery, The Johns Hopkins Hospital, Baltimore 5, Maryland.

945565—51——1

pH optimum of glucuronidase of male and female kidneys was determined. Sections of the renal cortex and medulla were assayed separately for glucuronidase activity. The influence of castration on renal glucuronidase activity was investigated in male and female mice of the dba strain.

MATERIALS AND METHODS

Mice of the C3H, A, dba, I, R III, CBA, and B alb C strains maintained at the National Cancer Institute were employed. The animals were segregated according to sex and offered an ad-libitum diet of Purina laboratory chow and water. Each animal was weighed, killed by cervical dislocation and the left kidney removed.[3] The perirenal fat was removed and the kidney rapidly weighed on a microtorsion balance. The entire kidney was then homogenized in 5.0 ml. of distilled water in an ice-bathed Potter-Elvehjem glass homogenizer. Aliquots of this homogenate were assayed for glucuronidase activity by the method of Talalay (*9*), with the modifications previously described (*1*). The homogenates of the adult dba kidneys were also assayed for total nitrogen content by a modification of Ma's micro-Kjeldahl method (*10*).

In 5 male and 5 female adult dba mice, portions of the renal cortex and medulla were assayed separately. Under a dissecting microscope a thin slice of the cortex was excised, weighed, and assayed for glucuronidase activity. A section of the medulla of the same kidney was then dissected out and assayed. The slices of cortex weighed approximately 50 mg., and the portions of medulla 20 mg. Male and female dba mice were castrated at 13 weeks of age. The animals were sacrificed, and their kidney glucuronidase activity was determined at 25 weeks of age.

The pH of optimum activity was determined for kidney glucuronidase obtained from male and female mice. The details of these assays are given in the footnote to text-figure 1.

Glucuronidase activity was expressed as: 1) micrograms of phenolphthalein liberated per milligram of moist tissue homogenized, or 2) micrograms liberated per milligram of total nitrogen in the tissue homogenate. The homogenates were incubated with 0.0005 M phenolphthalein glucuronide, pH 4.5, for exactly 1 hour at 37.5° C.

RESULTS

The kidney glucuronidase determinations in adult mice are summarized in table 1. As previously noted (*2*) the kidneys of the C3H and CBA mice exhibited considerably lower glucuronidase activity than the kidneys of mice of the other strains. In all strains, however, the kidney glucuronidase activity of the adult males was 1.3 to 2.6 times that of the adult females. The kidney glucuronidase activity of 10 adult male and 10 adult female dba mice was calculated per mg. of total nitrogen in the kidney homogenate as well as per mg. of moist weight of tissue homogenized. The details of these assays are given in table 2. On the basis

[3] In mice the left kidney is about 10 percent lighter than the right. The left was arbitrarily selected and was used throughout the experiments.

of moist weight, the activity of the males was 1.4 times that of the females. On the basis of total nitrogen, the ratio was 1.3:1.

TABLE 1. *Kidney glucuronidase activity of adult mice*

Strain	Number of animals		Mean kidney glucuronidase activity [1] ±1 standard error	
	Males	Females	Males	Females
C3H	10	10	0.71±0.08	0.30±0.01
A	10	10	4.3 ± .3	1.8 ± .1
dba	10	10	2.4 ± .1	1.7 ± .04
I	5	5	2.2 ± .2	1.6 ± .05
RIII	4	5	2.3 ± .2	1.6 ± .06
CBA	5	5	.36± .04	.28± .02
B alb C	4	5	3.9 ± .6	1.5 ± .07

[1] Micrograms of phenolphthalein liberated per mg. of moist kidney after incubation for 1 hour, pH 4.5, 37.5° C.

TABLE 2.—*Kidney glucuronidase activity of adult dba mice calculated on the basis of total nitrogen as well as moist weight*

Strain and sex	Mouse weight (gm.)	Weight left kidney (mg.)	Kidney glucuronidase activity	
			Micrograms phenolphthalein liberated per mg. moist kidney	Micrograms phenolphthalein liberated per mg. total nitrogen
dba male	20.5	144.4	2.3	82
"	22.5	159.8	2.3	84
"	20.5	134.8	2.2	78
"	24.5	175.0	2.8	90
"	22.0	146.0	2.2	78
"	24.0	158.8	2.5	89
"	23.5	159.4	2.1	73
"	19.5	119.0	2.9	99
"	21.0	143.2	1.9	67
"	22.5	167.4	2.4	92
Mean for dba males	22.0	150.8	2.4±0.1	83±3.0
dba female	20.0	116.0	1.8	65
"	18.0	106.0	1.6	57
"	19.5	107.4	1.7	66
"	16.0	92.0	1.7	59
	16.0	83.2	1.9	67
"	20.0	121.0	1.8	65
"	15.5	100.0	1.6	58
"	19.0	108.6	1.9	68
"	18.5	99.0	1.7	62
"	17.5	93.2	1.5	53
Mean for dba females	18.0	102.6	1.7±0.04	62±1.6

The kidneys of immature A, C3H, and dba mice of both sexes were assayed for glucuronidase activity. These animals were 5 to 7 weeks of age and weighed 12 to 16 grams. The results of these assays are given in table 3, where they are compared with the values obtained in adult animals of the same strains. The sex difference noted in adult mice was not demonstrated in immature animals.

945565—51——2

TABLE 3.—*Kidney glucuronidase activity of adult and immature mice*

Strain and age	Number animals assayed		Mean weight of mice (gm.)		Mean weight left kidney (mg.)		Mean kidney glucuronidase activity [1] ±1 standard error	
	Male	Female	Male	Female	Male	Female	Male	Female
dba, 11 weeks	10	10	22. 0	18. 0	151	103	2. 4±0. 1	1. 7±0. 04
dba, 5 weeks	10	10	15. 5	12. 3	98	76	1. 7± . 05	1. 7± . 05
A, 25 weeks	10	10	32. 5	26. 8	193	136	4. 3± . 3	1. 8± . 1
A, 7 weeks	5	5	18. 8	17. 6	106	98	2. 6± . 3	2. 2± . 1
C3H, 10 weeks [2]	10	10	25. 4	20. 9	180	129	. 59± . 1	. 33± . 02
C3H, 5 weeks	4	5	14. 5	12. 8	93	80	. 34± . 04	. 33± . 02

[1] Micrograms of phenolphthalein liberated per mg. of moist kidney after incubation for 1 hour, pH 4.5, 37.5° C.
[2] These C3H mice were of a different subline than those described in table 1. Also, the adult C3H mice in table 1 were 12 to 20 weeks of age.

The slices of kidney cortex exhibited higher glucuronidase activity than the pieces taken from the medulla. A summary of these determinations is presented in table 4. The cortex of the male kidneys showed greater activity than the cortex of the females, but the activity of the medullary tissue was not significantly different in the two sexes. The values for kidney cortex and medulla are not comparable to those obtained when whole kidneys were assayed because of the gross method of dissection and the minute portions of tissue assayed. The renal glucuronidase activity of intact male dba mice at 25 weeks of age was more than three times that of castrate males, intact females, and castrate females (table 5). The last three groups displayed essentially similar glucuronidase activity.

TABLE 4.—*Glucuronidase activity of the renal cortex and medulla*

Strain and sex	Kidney glucuronidase activity [1]	
	Cortex	Medulla
dba male	1. 3	0. 9
"	1. 9	1. 3
"	1. 6	1. 1
"	1. 7	. 2
"	1. 8	1. 2
Mean dba males	1. 7	. 9
dba female	1. 2	. 8
"	1. 6	1. 0
"	1. 5	1. 0
"	1. 4	1. 1
"	1. 3	1. 0
Mean dba females	1. 4	1. 0

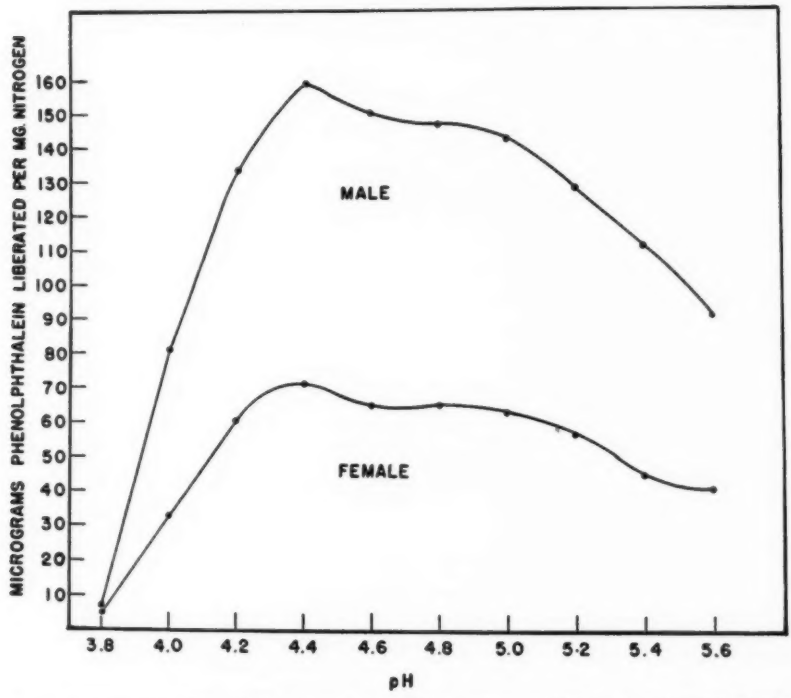
[1] Micrograms of phenolphthalein liberated per mg. of moist tissue after incubation for 1 hour, pH 4.5, 37.5° C.

The curves relating the activity of male and female kidney glucuronidase to pH are shown in text-figure 1. The enzyme from the kidneys of both sexes was most active at pH 4.4–4.6.

TABLE 5.—*Kidney glucuronidase activity of intact and castrated dba mice*

	Mean body weight (gm.)	Mean weight left kidney (mg.)	Mean kidney glucuronidase activity [1] ±1 standard error
Intact males	27. 3	227	5. 9±0. 96
Castrate males	24. 9	139	1. 6± . 07
Intact females	24. 0	130	1. 7± . 02
Castrate females	25. 8	130	1. 7± . 04

[1] Micrograms of phenolphthalein liberated per mg. of moist kidney after incubation for 1 hour, pH 4.5, 37.5° C.



TEXT-FIGURE 1.—pH-activity curves for kidney glucuronidase of male and female mice.

Both kidneys of an adult A male mouse and an adult A female mouse were removed and weighed. The kidneys of the male weighed 467.0 mg.; those of the female, 342.4 mg. Each pair of kidneys was homogenized in 25.0 ml. of distilled water. Aliquots of these homogenates were assayed in duplicate for glucuronidase activity and total nitrogen. The buffer of pH 3.8 was a phthalate-HCl mixture; all others were phthalate-NaOH mixtures.

DISCUSSION

The different glucuronidase activity observed in the kidneys of male and female mice is not unique. Several sex differences, morphologic and physiologic, have been described in the kidneys of mice. Kochakian (*11-13*) noted different alkaline phosphatase and arginase activity in the kidneys of normal male mice, castrated males, and castrated males given androgen. Dunn (*8, 14*) described a sex difference in the distribution of alkaline phosphatase in the kidneys of adult mice that was not apparent in newborn animals of the same strains.

Friedenwald and Becker (*15*),[4] studying a method for the histochemical localization of glucuronidase, noted that the glucuronidase of the rat kidney was maximal in the proximal convoluted tubules and decreased as the collecting tubules were approached. The slightly higher glucuronidase activity of the renal cortex as compared to the medulla suggests a similar distribution of the enzyme in the mouse kidney.

In the present experiments the kidney homogenates were routinely incubated at pH 4.5. Kerr (*4*) demonstrated two glucuronidase fractions with pH optima of 4.5 and 5.2 in kidney extracts from an unspecified strain of mice. In our experiments, however, two glucuronidase fractions were not demonstrated in homogenates of kidneys of male and female adult A mice.

In studies of liver glucuronidase previously reported from this laboratory (*1, 2*), no sex difference was noted in mice of the same strains employed in these experiments.

SUMMARY

Kidney glucuronidase activity was determined in adult and immature mice of seven inbred strains. In the adult animals the glucuronidase activity was 1.3 to 2.6 times greater in the males than in the females. This difference was apparent in assays based upon both the nitrogen content and the moist weight of the kidneys. No sex difference in kidney glucuronidase activity was apparent in immature mice. Castration of mature males was associated with a reduction in glucuronidase activity to the level of intact or castrate mature females. The pH of maximum activity was the same for kidney glucuronidase of male and female mice.

REFERENCES

(*1*) Morrow, A. G., Greenspan, E. M., and Carroll, D. M.: Liver glucuronidase activity of inbred mouse strains. J. Nat. Cancer Inst. 10: 657–662 (1949).

(*2*) ———: Comparative studies of liver glucuronidase activity in inbred mice. J. Nat. Cancer Inst. 10: 1199–1203 (1950).

(*3*) Eschenbrenner, A. B., and Miller, E.: Sex differences in kidney morphology and chloroform necrosis. Science 102: 302–303 (1945).

(*4*) Kerr, L. M. H., Campbell, J. G., and Levvy, G. A.: Beta-glucuronidase as an index of growth in the uterus and other organs. Biochem. J. 44: 487–494 (1949).

[4] Personal communication, Dr. Bernard Becker, Department of Ophthalmology, The Johns Hopkins Hospital, Baltimore, Md.

(5) GYÖRGY, P., SEIFTER, J., TOMARELLI, R. M., and GOLDBLATT, H.: Influence of dietary factors and sex on the toxicity of carbon tetrachloride in rats. J. Exper. Med. 83: 449–462 (1946).

(6) GOLDIN, A., GREENSPAN, E. M., GOLDBERG, B., and SCHOENBACH, E. B.: Studies on the mechanism of action of chemotherapeutic agents in cancer. I. A sex difference in toxicity to the folic acid analogue, 4–aminopteroylglutamic acid. Cancer 3: 849–855 (1950).

(7) WELLS, B. B., and KENDALL, E. C.: The influence of corticosterone and C17-hydroxydehydrocorticosterone (Compound E) on somatic growth. Proc. Staff Meeting Mayo Clinic 15: 324–328 (1940).

(8) DUNN, T. B.: Some observations on the normal and pathologic anatomy of the kidney of the mouse. J. Nat. Cancer Inst. 9: 285–301 (1949).

(9) TALALAY, P., FISHMAN, W. H., and HUGGINS, C.: Chromogenic substrates. II. Phenolphthalein glucuronic acid as substrate for the assay of glucuronidase activity. J. Biol. Chem. 166: 757–772 (1946).

(10) MA, T. S., and ZUAZAGA, G.: Micro-Kjeldahl determination of nitrogen. Indust. & Engin. Chem. (Analyt. Ed.) 14: 280–282 (1942).

(11) KOCHAKIAN, C. D.: The effect of castration and various steroids on the arginase activity of the tissues of the mouse. J. Biol. Chem. 155: 579–589 (1944).

(12) ———: The effect of various steroid hormones on the alkaline and acid phosphatases of the kidney of the mouse. Am. J. Physiol. 145: 118–122 (1945).

(13) ———: Histochemical study of alkaline phosphatase of the kidney following various methods of histologic fixation. Am. J. Physiol. 152: 257–262 (1948).

(14) DUNN, T. B.: Sex difference in the alkaline phosphatase distribution in the kidney of the mouse. Am. J. Path. 24: 719–720 (1948).

(15) FRIEDENWALD, J. S., and BECKER, B.: The histochemical localization of glucuronidase. J. Cell. & Comp. Physiol. 31: 303–310 (1948).

LEUKEMOID REACTIONS TO TRANSPLANTED MOUSE TUMORS[1]

JEANNE C. BATEMAN, M. D., *Research Fellow, Pathology Section, National Cancer Institute, National Institutes of Health, U. S. Public Health Service, Bethesda, Md.*

It was previously observed in this laboratory that a transplantable myoepithelioma of the mouse originating in the salivary gland produced a great enlargement of the spleen in the host, with extreme myelopoiesis in this organ (*1*). Since this observation, random blood counts on animals bearing this same type of tumor have revealed an extreme leukocytosis. The present investigation was undertaken, therefore, to study the factors influencing the development of this leukocytic reaction to transplanted myoepitheliomas. Three transplanted lines of this tumor type and, in addition, a fibrosarcoma that had been observed to cause a considerable increase in the size of the spleen were used in this study.

It was hoped that experimental methods could be devised to determine whether the leukocytosis was directly related to the growth of the tumor, or whether it was secondary to some contaminating organism that might either be transmitted with the tumor or that established itself in the favorable culture medium afforded by the tumor tissue. The general lines of investigation followed were: 1) Repeated blood counts on mice transplanted with the four tumor lines in an attempt to correlate the size and condition of the tumor with the number of granulocytes in the peripheral blood. 2) Observation of the effect of surgical removal of the tumor on leukocytosis. 3) Bacterial cultures from tumor tissue, with inoculation of any organisms isolated into other mice to determine whether such contaminating organisms independent of the tumors could be responsible for the leukocytosis. 4) Investigation into the effects on the leukocytosis of antibiotics that are potent against a wide spectrum of infecting organisms.

TUMORS STUDIED

Myoepithelial tumors have been found in inbred strains of mice carried at the National Cancer Institute and are frequent particularly in strains A and C. These tumors have been described briefly by Lippincott, Edwards, Grady and Stewart (*2*), and Andervont and Dunn (*3*).

Reports of an exact counterpart to this tumor have not been found in

[1] Received for publication July 7, 1950.

man, although Sheldon (*4*) has described several sweat-gland tumors in which myoepithelial elements predominated. In a later paper (*5*) he described mixed tumors of the salivary gland in which neoplastic myoepithelial cells played a prominent part. In the mouse, however, the myoepithelial cell in its various modifications apparently composes the entire tumor.

These tumors represent a distinctive type, and all the tumors examined, both original and transplanted, are of a fairly uniform structure. The appearance of these tumors is so characteristic that the correct identification can usually be made on gross examination. They nearly always arise in the region of the salivary glands, although they have rarely been found arising from the mammary gland. They rapidly become cystic and, when opened, the lining of the cyst appears rough and uneven, covered by a layer of necrotic tissue. The thickness of the cyst wall at different points varies from about 1 mm. to 8 mm. or more. The most distinctive feature of these tumors in the gross is the stringy, tenacious mucoid fluid that fills the cyst, and that can be scraped from the cut surface of portions of tumor tissue forming the cyst wall. The cystic center of the myoepitheliomas apparently results from necrosis, since necrotic cells are always found immediately adjacent to the cyst cavity and very small tumors are usually solid.

On microscopic examination the cells composing the tumor are pleomorphic (figs. 1, 2, and 3). They vary from an elongated, spindle shape with fusiform nuclei to an oval or round cell with a vesicular nucleus. The cytoplasm is more eosinophilic in the fusiform cells than in the rounded cells and is sometimes vacuolated. The tumors have a certain resemblance to fibrosarcomas, particularly in areas where fusiform cells predominate, but they are readily distinguished by the lack of uniformity in cell type. Cell groups in the myoepithelioma are indistinctly separated into compartments by bands of connective tissue (figs. 2 and 3). There is a suggestion of stratification in the arrangement of the cell forms, since the rounded cells tend to be located at the outermost margin, nearest the normal tissue surrounding the tumor or adjacent to the connective-tissue bands within the tumor. Ovoid cells which suggest a transition form may be discerned between the round and the fusiform cells. Mitotic figures are frequent in the round cells. Some of the cells with round nuclei seen in the section may be the result of transverse cutting of fusiform cells, as suggested by Sheldon, but they are not seen with the definite fascicular arrangement such as is found in fibrosarcomas.

Rarely these tumors have shown keratinization and indistinct pearl formations which at times resemble Hassall's corpuscles in the mouse thymus. Since the myoepithelial or basket cell is derived from an epithelial cell, this keratinization may be a possible derivation from the tumor cell, or it may represent another epithelial element in what may be an essentially mixed tumor. Studies previously carried out in this laboratory using the phosphotungstic-acid-hematoxylin stain have demon-

strated myofibrils in the fusiform cells. It is on the basis of this observation that this group of tumors has been diagnosed as myoepithelioma.

The fibrosarcoma was composed of large spindle cells with hyperchromatic ovoid nuclei. It resembled the fibrosarcoma that was described by Parsons (*6*), and that was considered to be associated with a myelogenous leukemia. The leukemoid reaction to the fibrosarcoma during the present study was never so extreme as that occurring in the myoepithelioma. Such a reaction has been reported with other fibrosarcomas (*7*).

The tumor lines studied (figs. 1–4) were:

Myoepithelioma A, which arose in a strain A mouse obtained from W. E. Heston. This tumor was transplanted subcutaneously into successive generations from the 9th to the 20th.

Myoepithelioma B, which arose spontaneously in a strain C mouse obtained from W. E. Heston. Tumor-bearing animals from the 9th to the 20th transplant generation were observed.

Myoepithelioma C, which arose spontaneously in a strain A mouse in this laboratory. Observations were made on tumor-bearing animals from the 15th to the 24th transplant generation.

Fibrosarcoma D, which was induced by the injection of approximately 0.3 mg. of methylcholanthrene in an aqueous suspension into the wall of the stomach of a strain C3H mouse without the milk agent. It grew rapidly when transplanted subcutaneously, and the mice developed a leukemoid reaction. This tumor was studied in animals of nine successive transplant generations from the 16th to the 25th.

GENERAL METHODS

All animals were maintained on Purina laboratory chow and water *ad libitum*. The mice were examined at weekly intervals, at which time the tumors were measured and ulceration and eschar formation were noted. Blood counts were done prior to tumor transplantation and subsequently at 2- to 14-day intervals. Hemoglobin determinations were made by an acid hematin method and read on the Coleman Junior spectrophotometer. Special calibrated pipettes[2] for small animals were used for red- and white-cell counts; the improved Neubauer counting chamber used in standard hematologic work was employed. Smears for differential counts were stained with MacNeal's polytetrachrome stain or the Giemsa stain. The Rees Ecker method was employed for platelet counts.

Tissue obtained at autopsy was fixed in Zenker-formol, embedded in paraffin, sectioned, and stained routinely with hematoxylin and eosin.

Bits of tumor tissue were implanted subcutaneously into the flank by means of a trocar. The transplants were made at 4- to 5-week intervals on each of the myoepitheliomas. The fibrosarcoma grew so rapidly that transplantation was necessary every 2 to 3 weeks.

[2] Arthur H. Thomas Co., Philadelphia, Pa.

OBSERVATIONS AND RESULTS

I. Tumor Growth

The transplanted myoepithelial tumors in all three lines first became palpable as small firm nodules at the site of inoculation after 1 to 2 weeks. As the tumor grew, it became soft and cystic. After a variable period there were ulceration and evacuation of the contents of the cyst. Following ulceration the tumors tended to shrink, leaving a deep, dry crater surrounded by a ridge of tumor tissue. Occasionally there was a metastasis to the regional axillary lymph node.

The transplanted fibrosarcoma followed a similar but more rapid course. These tumors tended to grow larger than did the transplanted myoepithelial tumors, and there was no cyst formation. Six mice bearing this tumor developed pulmonary metastases.

Control blood counts done immediately prior to tumor implantation revealed the range as well as the average for each strain of animals shown in table 1. As can be seen, the age range at the time of implant was wide. No correlation could be established between the white-cell count and the age in these animals.

Table 1.—*White-cell counts and age of mice at time of tumor implantation*

Tumor experiment	Strain	Number animals	White blood cells per cu. mm.[1]		Age in days	
			Range	Average	Range	Average
Myoepithelioma B	C	21	6, 500–26, 300	15, 100	37–222	97
Myoepithelioma A and Myoepithelioma C	A	70	4, 300–24, 500	11, 600	26–185	80
Fibrosarcoma 233	C3H	27	5, 600–27, 000	14, 870	32–253	100

[1] 1 count on each animal.

In all animals in which serial blood studies were done, a continuing rise in white-cell count was seen following tumor implantation. Gradual acceleration of leukopoiesis not infrequently increased to the point where the white-cell count doubled itself within a few days. The peak of hematopoietic activity usually occurred within the last week or so of life. In text-figure 1 the last white-cell count done on each animal is plotted against the time in days following tumor implantation. Since some of the final counts were done 2 to 3 weeks prior to death, it is probable that these figures are only a partial indication of the leukocytosis finally developing. However, it can be seen that many of the counts rose to extremely high levels (highest 912,000). A much higher average figure would probably have been reached if terminal records had always been available.

Differential counts revealed a reversal of the granulocyte-lymphocyte ratio normally found in mice (*8*). The percentage of metamyelocytes was increased, but myelocytes were found only in cases in which the white-cell count was 300,000 or more. Although granulopoiesis was most affected, there was an absolute increase in the lymphocytes and monocytes as well.

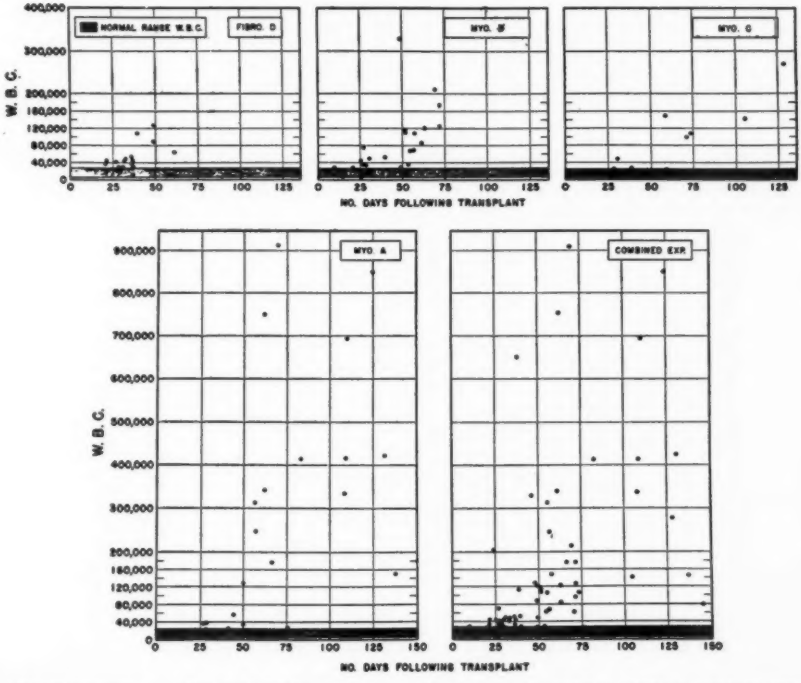
A moderately severe anemia developed in the majority of the animals, and an occasional normoblast appeared in the peripheral blood.

The platelet counts ranged from 560,000 to 1,167,000 in 8 animals that had white-cell counts of 64,000 to 340,000. One tumor-bearing mouse with a white-cell count of 16,100 had a platelet count of 450,000. Platelet counts on a group of 16 normal strain A and C mice including males and females (6 were 6 weeks old, 10 were 1 year old) ranged from 361,000 to 561,000. Association of increased numbers of granulocytes and platelets in the peripheral blood has been observed in man. It is also known that the platelet count may be greatly increased in myeloid leukemia (*9*).

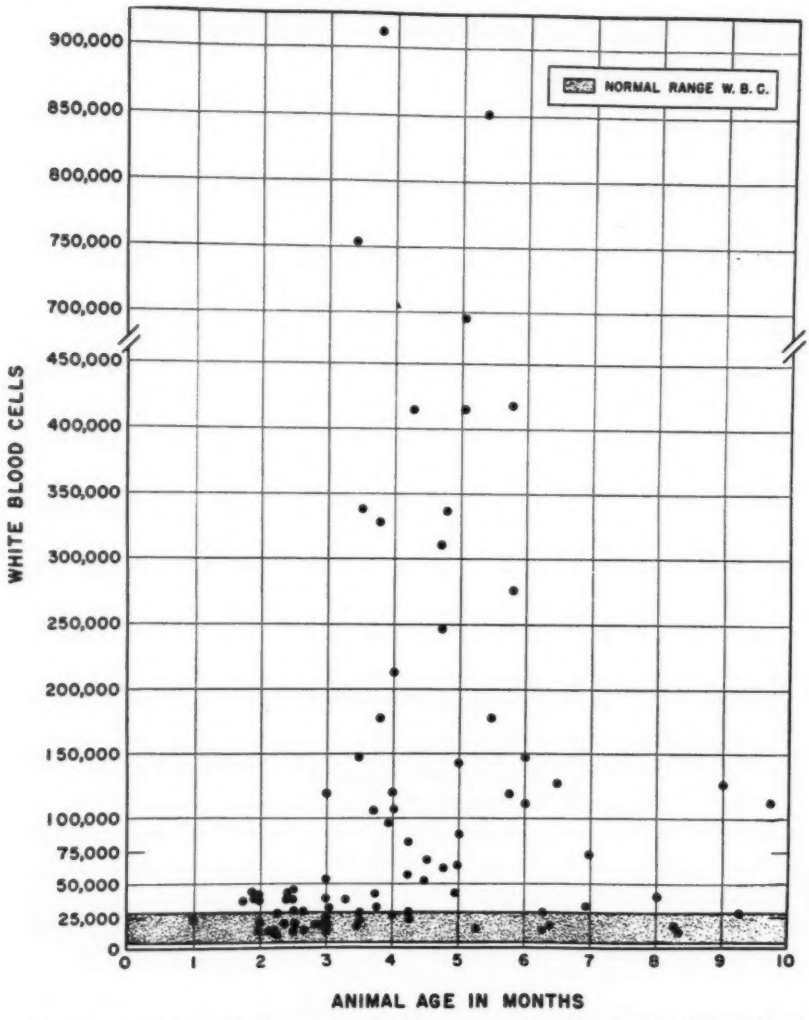
Coincident with the abnormal rise in the white-cell count, the spleen became palpable in all of the mice observed.

The following conditions were considered as possible factors influencing the elevation of white-cell counts, and their significance was analyzed statistically:

1. Duration of tumor implant (text-fig. 1).
2. Age of the expermental animal (text-fig. 2).
3. Size of the tumor and its condition, whether intact, ulcerated, or with eschar (text-fig. 3).

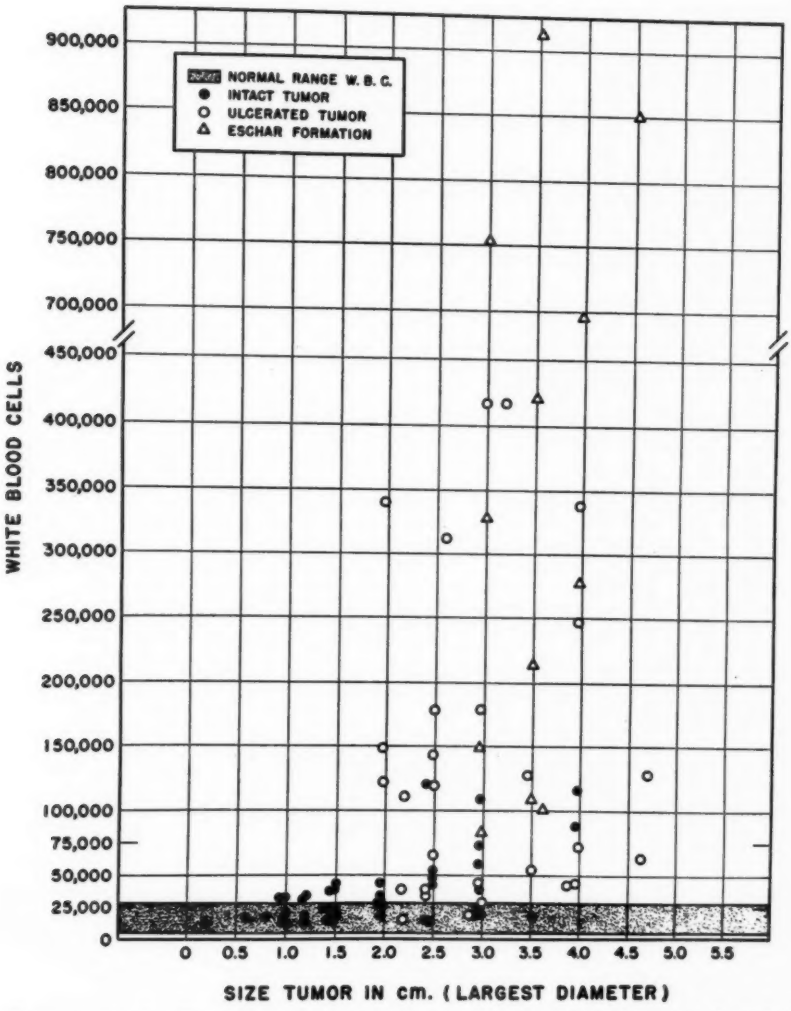


TEXT-FIGURE 1.—The last white-cell count done on each animal plotted against the time in days following tumor implantation. Each group of animals is given separately. A composite chart indicates the trend of the combined group.



TEXT-FIGURE 2.—The final white-cell count on tumor-inoculated mice in experiments Myoepithelioma A, Myoepithelioma B, Myoepithelioma C, and Fibrosarcoma D plotted against the age of the animal.

The statistical analysis of the data involved setting up a multiple correlation (analysis of variance) in an attempt to explain the total white-cell count as a result of some linear function of the three elements (duration of implant, size of tumor, and age of animal) given above. Total variation explained by combinations of these three factors was compared, separately for each combination of the factors. Where the adding of a factor significantly decreased the unexplained variation, the factor was considered definitely related to the final white-cell count. Where adding



TEXT-FIGURE 3.—The last white-cell count done on each animal is plotted against the size of the tumor at the time of the count. Intact tumors are indicated by solid circles, ulcerated tumors by open circles, and eschar formation by triangles. The four experimental groups are considered together.

a factor reduced the unexplained variation somewhat less, it was considered that the factor might be related to the white-cell count, but the evidence was not considered conclusive.

Statistical analysis of data presented in text-figures 1, 2, and 3 revealed definite indication that the elevation in count was related to the duration of implant and some indication that the size of the tumor and presence of ulceration also affected the white-cell count. The latter was not

clearly evident in all experiments. It was apparent that the duration of tumor growth and the size and condition of the tumor were related. Animals in different age groups showed no differences in the degree of leukemoid response to tumors.

II. Surgical Removal

Surgical removal of tumors was performed on 14 mice (table 2). It was not possible to remove the tumors in animals having very large tumors and correspondingly high white-cell counts. Three animals (2211, 1011, and 316) had normal white-cell counts at the time of operation. Surgery produced a transient small rise in their counts. In the remaining 11 tumor-bearing mice with leukocytosis, surgical removal of the tumor resulted in a fall in the white-cell count by the 6th to the 20th day. The count was still falling when evidence of tumor recurrence appeared in some of the animals. Return of leukocytosis eventually took place in all animals that exhibited recurrence of tumor. The 3 mice (2211, 1011, and 316) without initially elevated white-cell counts, and 1 mouse (3119) with a small tumor initially, did not develop recurrence by the end of 6, 8, 9, and 9 months, respectively; and their white-cell counts remained normal.

III. Bacteriologic studies

Smears and cultures for the detection of bacteria were made from the tumor tissue of five animals killed for this purpose, as well as from material obtained by aspiration of tumors in a group of three live mice. Although all of the tumors in the first group of animals that were killed showed some degree of ulceration, a pleomorphic gram-positive diplococcus growing in chains and clumps was obtained from the cultures of three of the five examined. Broth suspensions of this organism injected into five healthy mice of the same strain as the one from which the organism was isolated produced a transient minimal rise in the white-cell count (13,200 to 20,900) in only one animal. An organism identified as *Proteus vulgaris* was isolated from the tumor aspirate of one mouse in the live group; there was no growth in the cultures from the other two mice in this group. Thus, no specific organism was found by these methods to account for the leukocytosis associated with tumor growth.

Adequate virus studies were not done. Reports on virus infection of tumors (*10, 11*) indicate that changes in the leukocyte count resulting from a transmitted virus occur within a 2-week period after inoculation and do not coincide and increase with the tumor growth.

IV. Effect of antibiotics

Aureomycin is an antibiotic with an unusually wide spectrum of effectiveness against bacteria, rickettsia, and virus pathogens (*12–14*). Five animals having white-cell counts of 17,000, 82,700, 120,700, 128,000, and 754,600, respectively, and tumors ranging from 2 to 3 cm. in diameter were treated with aureomycin. The drug was administered subcutaneously

in daily doses of 2 mg. for periods ranging from 1 to 4 days, inclusively. There was no significant effect on the white-cell count, the appearance of the tumor, or the clinical course in these animals.

TABLE 2.—*Course of white-cell count following surgical removal of tumor*

Tumor experiment	Mouse No.	Control W. B. C.[1]	Preop. W. B. C.	Age tumor (days)	Postoperative		Remarks[2]
					W. B. C.	day	
Myoepithelioma A	2113	5, 900	24, 100	29	8, 700	6	No tumor.
					7, 700	35	Tumor 2.0 cm.
					42, 700	58	Tumor 4.0 cm.
Myoepithelioma A	2110	14, 300	38, 500	28	17, 300	6	No tumor.
					11, 300	20	Tumor 0.2 cm.
					26, 700	37	Tumor 1.0 cm.
Myoepithelioma A	2012	10, 000	38, 100	28	11, 800	14	No tumor.
Myoepithelioma A	2211	--------	14, 000	27	20, 100	24	No tumor.
					14, 500	120	"
Myoepithelioma B	1113	19, 600	42, 800	31	32, 000	6	No tumor.
					37, 000	18	Tumor 2.0 cm.
Myoepithelioma B	1210	8, 500	32, 000	28	12, 800	6	No tumor.
					21, 100	20	Tumor 0.5 cm.
					35, 000	36	Tumor 1.0 cm.
					137, 700	65	Tumor 3.0 cm.
Myoepithelioma B	1215	6, 600	38, 800	25	23, 800	6	No tumor.
					14, 800	12	"
					8, 300	19	Tumor 0.2 cm.
					59, 000	46	Tumor 3.0 cm.
Myoepithelioma B	1115	8, 600	41, 200	25	18, 300	6	No tumor.
					11, 600	12	Tumor 0.2 cm.
					7, 850	20	Tumor 0.4 cm.
					82, 300	46	Tumor 4.0 cm.
Myoepithelioma B	1011	--------	20, 300	28	23, 400	23	No tumor.
					13, 500	65	"
Myoepithelioma C	219	11, 500	45, 700	30	13, 600	4	No tumor.
					8, 800	15	Tumor 0.2 cm.
					18, 200	33	Tumor 1.0 cm.
					281, 000	139	Tumor 4.0 cm.
Myoepithelioma C	118	11, 500	24, 100	29	18, 200	16	Tumor 1.2 cm.
Myoepithelioma C	316	8, 700	19, 830	28	21, 800	6	No tumor.
					33, 200	20	Abscess.
					18, 000	168	No tumor.
					10, 200	248	"
Fibrosarcoma D	3017	27, 000	41, 600	23	9, 700	6	No tumor.
					27, 300	28	Tumor 1.5 cm.
Fibrosarcoma D	3119	20, 800	42, 100	48	24, 000	6	No tumor.
					16, 000	34	"
					14, 000	139	"

[1] White blood count at time of tumor implantation.
[2] All tumors seen were local recurrences in region of surgical scar.

Because of the effectiveness of streptomycin in Proteus infections (*15–17*) another three mice were given a 2.5-mg. dose of this drug daily by the subcutaneous route. When at the end of 5 days no response was apparent, 2 mg. of aureomycin was given in addition. The white-cell counts continued to rise steadily, and the animals died 6, 25, and 27 days following institution of therapy.

PATHOLOGIC FINDINGS IN TUMOR-BEARING MICE WITH A LEUKEMOID REACTION

The gross changes were similar to those generally described in mice showing leukemoid reactions (*18*); the only notably consistent change found in the tumor-bearing mice was splenomegaly, which was generally related to the degree of leukocytosis. Spleens from animals with markedly elevated white-cell counts varied from 2 to 3.2 cm. in length by .7 to .9 cm. in diameter.

Microscopical Findings

Bone marrow section (fig. 5) showed an increased proportion of the granulocytic cell series, even when the peripheral counts were only slightly elevated. The majority of the granulocytic cells were relatively mature "ring" forms.

Extramedullary hematopoiesis in the spleen was generally proportional to the leukocytosis of the peripheral blood. It was especially pronounced in animals with white-cell counts of 100,000 or more, and the predominant cell forms were of the granulocytic series. There appeared to be an increase in megakaryocytes (fig. 6).

The livers in mice with extreme leukocytosis showed myelopoietic tissue, especially in the portal areas (fig. 7).

The lungs showed a great number of granulocytes in the walls of the alveoli, and many granulocytes were present in the large vessels. Many of these showed pyknosis (fig. 8).

The tumors (figs. 1–4) showed the histologic structure previously described. There was a moderate infiltration with granulocytes in the tissues at the margin of some of the tumors, and some necrotic granulocytes in the necrotic tissue in the center. However, this was no more than is customarily seen in transplanted tumors of other types, after prolonged growth. There was never the great accumulation of leukocytes that may be found in frankly infected tumors.

DISCUSSION

Various tumors in both man and animals are known to produce an effect on other organs or systems. Most easily understood are the changes caused by neoplasms of endocrine organs, such as adrenal cortical tumors, pheochromocytomas, and adenomas of the islets of Langerhans. Other reactions are more difficult to understand, such as the altered protein metabolism in multiple myeloma, and the hypervolemia associated with ovarian tumors in mice described by Furth (*19*). The leukemoid reaction developing from the growth of certain tumors in mice, particularly the

myoepitheliomas, appears to be another instance of such a systemic reaction to a neoplasm. Absolute proof of such an association is always hard to furnish, but in the present study the increasing granulocytosis accompanying the growth of the tumor, the reduction or disappearance of this reaction after surgical removal of the tumor, and its recurrence along with recurrent growth of the tumor seemed convincing. The influence of such secondary factors as necrosis and possible bacterial infection of the tumor could not be entirely ruled out. Previous experience with tumors showing extensive necrosis has not revealed the extreme leukocytosis found with the myoepitheliomas. The variable results obtained on bacterial culture, the absence of a pronounced leukocytosis after injection of bacterial culture material, and the failure of large doses of potent antibiotics to reduce the leukocyte count suggest that secondary bacterial infection played at most a small role in the leukemoid reaction.

In this laboratory, myoepitheliomas in particular have produced extreme degrees of leukemoid reaction. Mammary tumors and fibrosarcomas in mice are also known to produce this effect, and a mixed-cell sarcoma in hamsters has been reported that produces extramedullary hematopoiesis and splenomegaly (*20*). This reaction on the part of the myoepitheliomas cannot be explained on the basis of any known functional relationship between the myoepithelial cell and a granulocyte-promoting factor. The fact that many other tumors produce this effect indicates that it is not specific.

It is apparent that certain tumors elicit a peculiar hematologic effect in mice and in other animals. A similar reaction in a few human cases has been observed (*21*, *22*). Although in this study the leukocytosis appeared to be related to the duration of the transplant and the size and condition of the tumor, the mechanism of this phenomenon remains to be explained.

SUMMARY AND CONCLUSIONS

1. Leukemoid reactions produced by four different transplanted tumor lines, including three myoepitheliomas and one fibrosarcoma, were studied.

2. The degree of leukocytosis was directly related to the duration of the transplant. The size of the tumor also appeared to be of some significance.

3. The age of the animal did not modify the leukemoid response to the tumor.

4. Surgical excision of the tumor reduced the white-cell count in 11 mice with leukocytosis. Reappearance of the tumor in 10 of the mice was followed by increasing granulocytosis.

5. Bacteria were isolated from some of the tumors. Only a mild leukocytosis could be induced in normal mice with cultures of bacteria isolated from tumors.

6. Limited studies with aureomycin and streptomycin suggest that these antibiotics have no effect on the leukemoid reaction to the tumors observed.

945565—51——3

UNIVERSITY OF MICHIGAN LIBRARIES

ACKNOWLEDGMENT

Grateful acknowledgment is made to Dr. Thelma Dunn for her help on pathology, to Dr. Birdsall N. Carle for bacteriological assistance, and to Mr. Marvin Schneiderman for statistical analysis of the data.

REFERENCES

(1) MAVER, M. E., and DUNN, T. B.: Catheptic activities of neoplasms and of the tissues of normal and tumor-bearing mice correlated with histologic changes. J. Nat. Cancer Inst. 6: 49–60 (1945).

(2) LIPPINCOTT, S. W., EDWARDS, J. E., GRADY, H. A., and STEWART, H. L.: A review of some spontaneous neoplasms in mice. J. Nat. Cancer Inst. 3: 199–210 (1942).

(3) ANDERVONT, H. B., and DUNN, T. B.: Attempt to detect a mammary tumor agent in strain C mice by X-radiation. J. Nat. Cancer Inst. 10:1157–1189 (1950).

(4) SHELDON, W. H.: The myoepithelium in sweat gland tumors. Distribution, histology, embryology and function. Arch. Path. 31: 326–337 (1941).

(5) ———: So-called mixed tumors of the salivary glands. Arch. Path. 35: 1–20 (1943).

(6) PARSONS, L. D.: Leukemia coincident with and transmissible by a spindle-celled sarcoma in the mouse. J. Path. & Bact. 40: 45–54 (1935).

(7) LEWIS, M. R.: Myeloid infiltrations occurring in the adrenals of animals bearing certain tumors. Am. J. Cancer 30: 95–101 (1937).

(8) STAFF OF THE ROSCOE B. JACKSON MEMORIAL LABORATORY: Biology of the laboratory mouse. Blakiston Co., Philadelphia (1941).

(9) WINTROBE, M. M.: Clinical hematology. Lea & Febiger, Philadelphia (1946).

(10) DEBRUYN, W.: A lymphopenia-causing agent, probably a virus, found in mice after injection with tumor tissue and with cell free filtrates of lymphosarcoma T 86157 (M B). Cancer Research 9: 395–397 (1949).

(11) TAYLOR, M. J., and MACDOWELL, E. C.: Mouse leukemia. XIV. Freeing transplanted line I from a contaminating virus. Cancer Research 9: 144–149 (1949).

(12) DUGGAR, B. M., *et al.*: Aureomycin—a new antibiotic. Ann. N. Y. Acad. Sc. 51: 177–342 (1948).

(13) BRYER, M. S., SCHLENBACK, E. B., CHANDLER, C. A., BLISS, E. A., and LONG, P. H.: Aureomycin. J. A. M. A. 138: 117–119 (1948).

(14) SPINK, W. W., and YOU, E. M.: Aureomycin: present status in the treatment of human infections. J. A. M. A. 141: 964–966 (1949).

(15) ABRAMS, H. L.: Septicemia due to Proteus vulgaris. New England J. Med. 238: 185–187 (1948).

(16) COMMITTEE ON CHEMOTHERAPEUTICS AND OTHER AGENTS, NATIONAL RESEARCH COUNCIL: Streptomycin in treatment of infections, report of 1000 cases. J. A. M. A. 132: 4–11 & 70–77 (1946).

(17) POOL, T. L., and COOK, E. N.: Present concepts of treatment of infections of urinary tract. J. A. M. A. 133: 584–587 (1947).

(18) BARNES, W. A., and SISMAN, I. E.: Myeloid leukemia and non-malignant extramedullary myelopoiesis in mice. Am. J. Cancer 37: 1–35 (1939).

(19) FURTH, J.: Hypervolemia secondary to grafted granulosa-cell tumor. J. Nat. Cancer Inst. 7: 103–113 (1946).

(20) KELSALL, M. A.: Hematopoiesis in the spleen of tumor-bearing hamsters. J. Nat. Cancer Inst. 10: 625–629 (1949).

(21) KARNOFSKY, D. A., ABELMAN, W. H., CRAVER, L. F., and BURCHENAL, J. H.: The use of nitrogen mustards in the palliative treatment of carcinoma. Cancer 1: 634–656 (1948).

(22) BATEMAN, J.: Unreported observations.

PLATES

945565—51——4

UNIVERSITY OF MICHIGAN LIBRARIES

PLATE 75*

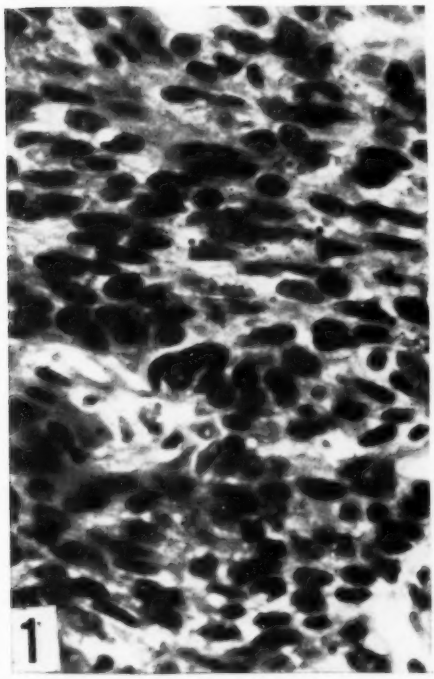
FIGURE 1.—Myoepithelioma A. Note different forms of tumor cells—a rounded form generally found adjacent to connective tissue septa and a fusiform type with vacuolation of cytoplasm. Hematoxylin and eosin stain. × 500.

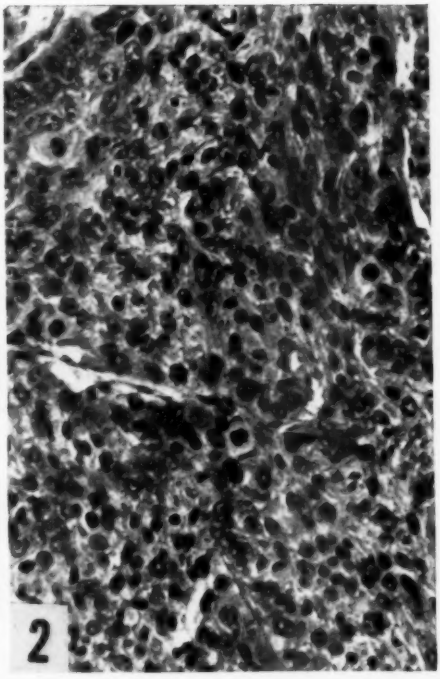
FIGURE 2.—Myoepithelioma B. Note pleomorphism of cells and frequent mitotic figures. Hematoxylin and eosin stain. × 300.

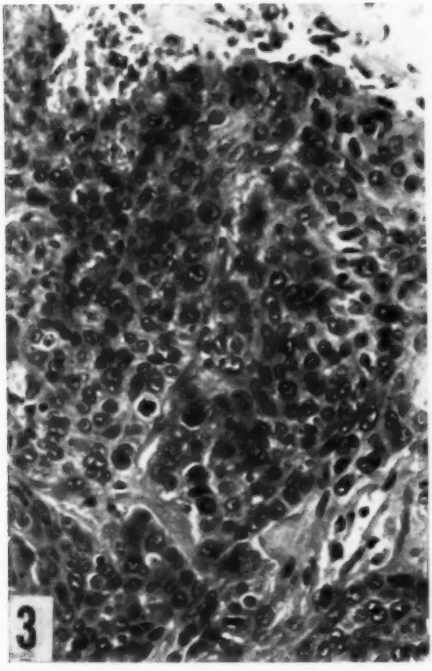
FIGURE 3.—Myoepithelioma C. Note characteristic mucoid material in central cyst margin shown at upper right. Hematoxylin and eosin stain. × 300.

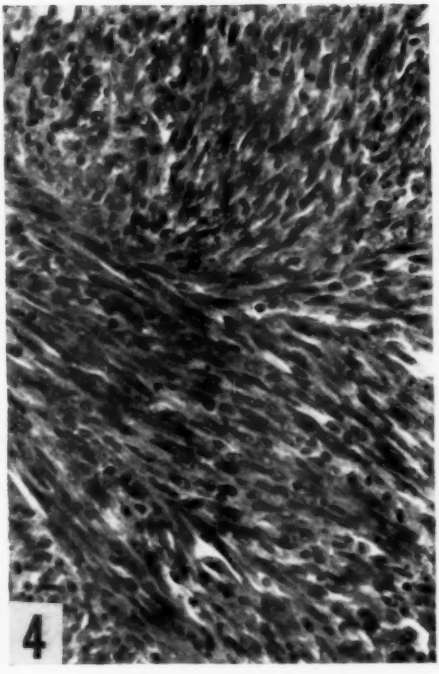
FIGURE 4.—Fibrosarcoma D. Note fascicular arrangement of elongate fibroblastic cells. Hematoxylin and eosin stain. × 300.

* Histology of tumors producing a leukemoid reaction. Note histological similarity in three myoepitheliomas.









929548—51——5

UNIVERSITY OF MICHIGAN LIBRARIES

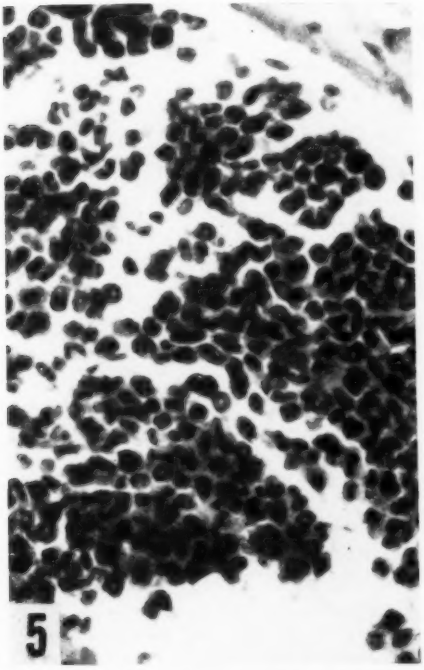
PLATE 76

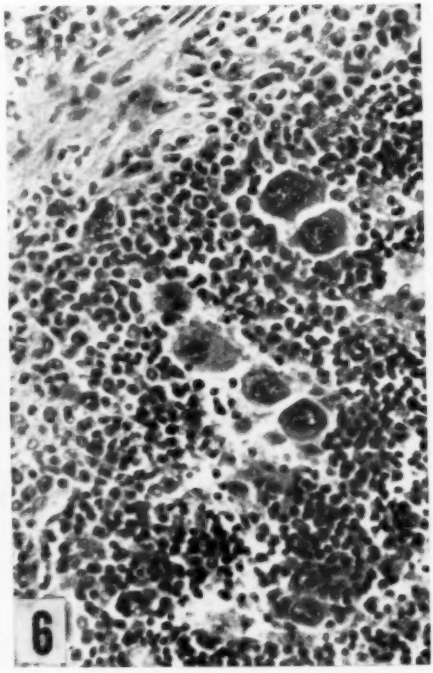
FIGURE 5.—Bone marrow. Predominance of granulocytic-cell types. Many "ring" forms are seen and relatively few erythropoietic cells. Hematoxylin and eosin stain. × 600.

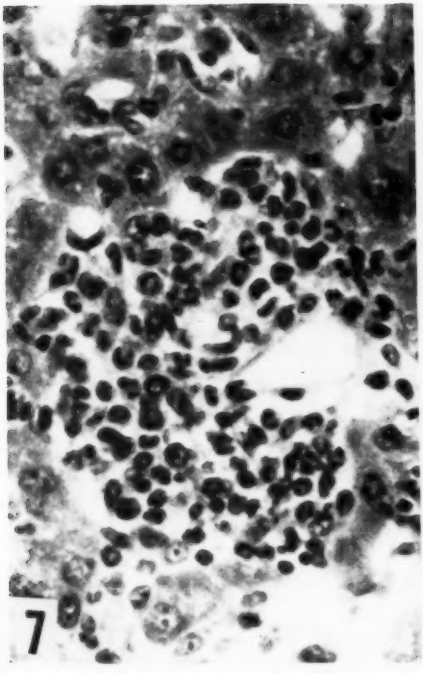
FIGURE 6.—Spleen. Extramedullary hematopoiesis, predominantly granulocytic. Capsule of spleen is shown at upper left; note number of "ring" forms immediately beneath it. Megakaryocytes appear to be increased. Hematoxylin and eosin stain. × 300.

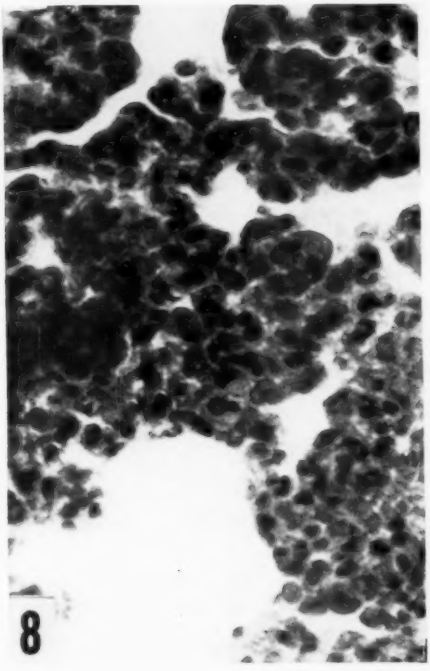
FIGURE 7.—Liver. Area of extramedullary hematopoiesis, predominantly granulocytic. Liver sinusoids also contain many cells of this type. Hematoxylin and eosin stain. × 60.

FIGURE 8.—Lung. Capillaries in alveolar wall containing many granulocytes, which are often pyknotic. Alveolar spaces are empty. Hematoxylin and eosin stain. × 300.









UNIVERSITY OF MICHIGAN LIBRARIES

A STUDY OF THE "GRUSKIN SEROLOGICAL TEST" FOR THE DETECTION OF CANCER; THE PRECIPITATION OF BLOOD PROTEINS BY ALCOHOL [1] [2]

N. HOLMGREN, M. S., R. W. DENTON, M. S., M. D., S. A. LEVINSON, PH. D., M. D., A. C. IVY, PH. D., M. D., and L. E. GRUBGELD, M. S., *Department of Clinical Science, University of Illinois College of Medicine, Chicago, Ill.*

INTRODUCTION

Many serological tests for the detection of cancer have been reported (*1*, *2*, *3*). With the exception of a few being presently studied, the proposed tests have either been discarded or tabled without further study, "false positive tests" being the major difficulty.

It appears that many investigators have seen the vital need for such a test, but no one has persisted in carrying any test beyond the early, more or less empirical stages of development. Among the various tests, that of Fuchs (*4*, *5*, *6*) has received the most confirmation and has been claimed to be 90 percent predictive.

It is common knowledge that the serological tests for syphilis were initially inaccurate, but in spite of this and their scientific nonspecificity, they have been developed by trial and error until they have become important in diagnosis. For example, Kahn (*7*) refers to the early "era of low serologic sensitivity" for the precipitin and flocculation tests for syphilis, which transpired from the first reports by a few German experimenters around 1907 up to his work in the early 1920's. Only in 1925, after 15 years of work, had optimal technical conditions been developed so that the Kahn test could realize an accuracy comparable to that of the Wassermann test. The official reports of International Conferences during Kahn's "era of high sensitivity" (*7*) shows a long period during which the sensitivity was developed from 47.6 percent in 1928 to 92.8 percent in 1935, while the specificity was maintained near 100 percent.

By analogy to the Kahn and similar tests, one might class the serological tests for the detection of cancer as being in an early stage of development, possibly in a period of low specificity; whereas the early syphilitic tests had low sensitivity.

[1] Received for publication September 11, 1950.
[2] Assisted in part by a grant from the Benjamin Gruskin Fund of the Lakeland Foundation, Chicago, Ill.

929548—51——6

UNIVERSITY OF MICHIGAN LIBRARIES

In this paper we wish to present some work directed toward an evaluation of the Gruskin serological test for cancer (*8*). This test was chosen for study because of its similarity in technique and possible basis of action to the now successful flocculation test for syphilis, because of its technical simplicity and the ready availability of materials for the test, and because its author has claimed for it an accuracy at least as good as others have reported for any cancer test.

The test depends upon obtaining a flocculation in the serum of cancer patients. Other tests for cancer, for example, Kahn's "Albumin A" fraction decrease (*9*, *10*), Kopaczewski's gelation rate increase with lactic acid (*11*, *12*), Ascoli and Izar's meiostagmin reaction (*13*), and many others (*14*, *17*), have shown aberrancies in the chemical or physical properties of cancer blood; hence it is not surprising that a colloidal instability may exist as claimed for the Gruskin test.

MATERIALS AND METHODS

Preparation of Gruskin "antigen".—The liver is removed from Bovine embryos up to 2 months of age taken at the time of slaughter. The embryonic liver cells are washed thoroughly in saline, the wet tissue extracted with acetone, and the cells dried on a Buchner funnel at room temperature. The dried cells are then arbitrarily diluted with 10 cc. of absolute alcohol per gram. The alcohol is allowed to remain on the cells and age for a period of 3 weeks or longer with occasional shaking.

Type and preparation of blood serum samples for testing.—Blood was drawn from cancer and noncancer subjects in the morning before breakfast after a 12-hour fast. There were no dietary restrictions the day before unless otherwise specified. The serum was recorded (as a discard) but the results were not included in the total results if the serum was lipemic, icteric, or hemolyzed. The presence of jaundice, edema, or high fever was noted, since Dr. Gruskin noted that these conditions rendered the test unreliable. He also reported that radiological treatment or treatment with stilbestrol rendered the results unreliable. Later, the blood of patients with diagnosed, untreated cancers of any organ was employed.

As specified in the original test, the specific gravity of each serum was determined by the falling-drop method and 0.5 cc. of each serum was treated with that amount of 0.85 percent NaCl solution required to make the specific gravity the same as that of the lowest of the sera to be examined that day. (We believe that this is irrational, and that to be rational the specific gravity of the sera should be equated with that of the "control normal serum" used in the test.) The serum was inactivated at 56° C. for 30 minutes.

Performance of the test.—In accordance with the original directions, the test is performed at room temperature. The serum is carefully layered drop by drop over an amount of the alcoholic solution of the "antigen" (usually 0.03 cc.) found by previous titration with a known cancer patient and known normal patient. The titration end-point

is the amount of "antigen" that gives flocculation with the cancer serum and no flocculation with the noncancer serum. The method of addition of serum to "antigen" is important. The serum is added more slowly at first, the addition of the 0.5 cc. taking approximately 30 to 45 seconds. If the whole 0.5 cc. of serum is added at once, no flocculation takes place.

A precipitate always forms at the contact of the serum with the alcoholic solution of the "antigen." After standing 3 minutes with the alcohol-serum interface undisturbed, the tubes are shaken for 2 minutes and then 0.5 cc. of 0.85 percent NaCl is added. After waiting for 1 minute, the tubes are again shaken for 2 minutes. Another 0.5 cc. of the 0.85 percent NaCl is added, and the results of the test are read at various intervals by looking for flocculi—"amorphous white plates instead of globules or particles." This distinction is difficult to make, since most tests give combinations of the two types of floccules.

No standard time of reading the results had been established by Gruskin. Since it was found that almost all sera gave some degree of flocculation on the immediate reading, Gruskin reported that the flocculi of false-positive reactions would redissolve on (a) standing at room temperature, (b) standing overnight at refrigerator temperature, and (c) on further dilution with saline. A combination of readings was therefore taken, the first at 1 minute after the test was completed, the second at 1 hour, and the third after overnight-standing in the refrigerator. Readings two and three were made after we had gained experience during the first 3 months of study, during which period we were occasionally supervised by Dr. Gruskin. These readings were recorded individually, in order to ascertain which was the most reliable. For statistical purposes, and for other reasons indicated later, a certain reading was assigned a numerical value.

Method of Reading.—Results are recorded from negative to ++++, according to size and quantity of the floccules. Doubtful, or ±, indicates that the test was not completely free of flocculation although a granular appearance was present. The value for a positive or negative test is indicated in each experiment. If not stated, a one plus test was considered positive for "cancer" as directed by Gruskin.

The person performing the test did not know the source of the serum.

Chemical observations on the antigen.—It was desirable to know something of the nature of the substance in the alcohol that Gruskin has termed "antigen," because if the substance should be essential for the reaction, a knowledge of its chemistry would help prepare batches with the same "potency."

The "Gruskin antigen" was prepared as follows: The livers of calf embryos under 2 months of age after washing with saline were triturated by gyrating and rotating in a small quantity of normal-saline solution. This pulpaceous mass was washed with acetone, and the remaining dried material was allowed to stand with alcohol for 3 weeks prior to use. Thus, the "antigen" is not soluble in acetone, but is soluble in absolute alcohol. Dr. L. L. Gershbein then made a chemical study of the "antigen."

UNIVERSITY OF MICHIGAN LIBRARIES

Analysis of the alcoholic "antigen" solution—ultraviolet absorption spectrum.—The "aged antigen" alcoholic solution was filtered twice, and the absorption spectrum was determined in the ultraviolet region by means of a Beckman quartz spectrophotometer. A continuous spectrum was obtained, which is incompatible with a proteinaceous solution but compatible with a phospholipid solution.

Amino acids and proteins.—The ninhydrin, Biuret, and Hopkins-Cole tests, even after acid hydrolysis, were negative.

Analysis of solids.—A 19.5-ml. sample of the stock alcoholic solution of "antigen" was concentrated under vacuum below 33° C., and 80 mg. of "phospholipid" matter was obtained. This was extracted with anhydrous ether and 32 mg. of a white solid remained insoluble. Analysis (Holk) of the ethereal solution gave the following results: phosphatide phosphorus, 1.27 mg.; KOH hydrolyzable phosphatide phosphorus, 1.232 mg.; sphingomyelin phosphorus by difference, 0.038 mg.; KOH hydrolyzable phosphatide choline, 2.8 mg.; total phosphatide, 32.62 mg.; sphingomyelin, 0.98 mg.; lecithin, 18.48 mg.; cephalin, 13.16 mg.

The white residue contained a further amount of phospholipid. It was completely devoid of proteinaceous material.

Soxhlet alcoholic extraction of the liver powder dried with acetone yielded: sphingomyelin, 9.55 mg.; lecithin, 25.4 mg.; cephalin, 29.30 mg.; or a total phospholipid of 64.25 mg. from 2.93 gm. of the dried liver powder. An analysis for cholesterol was not attempted. The preparation made by the alcoholic extraction reacted with serum as the "antigen" prepared by the Gruskin method.

A significant difference in the chemistry and the reaction of the product in the Gruskin test did not occur whether the alcoholic solution was made by letting the powder stand with alcohol for 3 weeks or by Soxhlet extraction for 24 hours.

The chemical study showed that the "Gruskin antigen" is a non-proteinaceous mixture high in phospholipids, especially lecithin and cephalin. A few tests were made on the serum of cancer patients with an alcoholic solution of lecithin and cephalin and results similar to those with the "Gruskin antigen" were obtained. The cephalin solution used in performing the cephalin-flocculation test in liver disease yielded negative results.

It might be added that Sachs (*17*) in 1932 employed an alcoholic-lecithin (1 percent) solution in a serodiagnostic test for cancer.

Alcoholic extracts were made of acetone-dried embryonic liver, with and without previous autolysis and with and without desiccation before extraction with alcohol. The serodiagnostic results were the same. Soxhlet extraction was conducted on embryonic liver with alcohol, then ether, and then the residue was extracted with warm water. Flocculation was obtained only with the alcoholic extracts. The results of these and several other procedures, which need not be mentioned, showed that alcohol was an essential part of the extraction procedure applied to the

liver tissue and of the reaction, since an alcoholic solution of antigen was required to give the reaction.

RESULTS

Predictive value of the Gruskin test.—In this *first phase of the investigation* the test was applied, following carefully Dr. Gruskin's instructions concerning technique, to over 100 patients having proved carcinoma, and to over 100 individuals not having carcinoma (12 months' follow-up). Of the cancer cases, approximately three-fourths (76 percent of 124) had had some types of therapy for cancer (usually surgical removal or X-radiation of the tumor) for various lengths of time before the blood serum was obtained for testing. The remainder of the cases were relatively early or had extensive metastases in spite of previous treatment. Of the noncancer patients one-half (101 of 202) had had some sort of illness consisting of various degrees of inflammation or metabolic dysfunction. The remaining controls were relatively young medical students and technicians with no physical complaints. The heterogeneous group was formed in order to examine the test first under conditions of medical practice, or under conditions where differentiation would have to be made between the sick without and with carcinoma, and not between presumptively healthly individuals and the obviously cancerous subjects.

It was found that 57 percent (115 of 202) of the noncancer cases gave "false positive" reactions, and that only 8 percent (10 or 124) of the cancer cases gave "false negative" tests. When these results (table 1) were examined by the chi-square test, it was found that the difference observed could occur by chance less than 1 in 100 such groups. Thus, *the test has predictability value for cancer or something associated with cancer*; however, the high incidence of "false positives" invalidates the test for diagnostic purposes.

It was found further that the first reading (1 minute) had greater predictive value than the second (1 hour) and the third (24 hours), and that an appraisal of the group of readings was of more predictive value than one alone. This appraisal was subjective and of value only after much experience in reading the test. Sufficient tests were made at this time on *pooled serum* from cancer and noncancer subjects to demonstrate that the results of the test were consistently reproducible. This was further evidence that on the average there was a detectable difference between the serum of cancer- and non-cancer-bearing persons.

Tests were performed on a total of 373 non-cancer-bearing subjects (patients and normal subjects), the "false positives" being 45 percent, and on a total of 273 cancer-bearing subjects, the "false negatives" being 5 percent.

Unfortunately the foregoing portion of this study was performed before we studied the effect of temperature on the test and the test-retest reliability of the test. In view of the consistent differences found in the pooled sera from cancer and noncancer patients, it was thought best to determine whether the test had predictive value when applied to individual sera. It would have been better to have reversed this experimental plan.

TABLE 1.—*Comparison of results in cancer cases of all types*

Type of case	Positive test	Negative test
A. Known cancer—no therapy—"fresh" cases	28	[1] 5
B. Recurrence from metastases though treated previously	15	
C. Known cancer—treatment in progress or completed less than 5 years	45	[2] 4
D. Known cancer—operated or X-rayed, and blood taken within 2 weeks after therapy	12	[3] 1
E. Known cancer—5-year cures	14	
	114	[4] 10(or 3)
Total cancer cases of all types		124
F. Medical-student and nonpregnant-women controls	39	62
G. Noninflammatory patients—controls	19	8
H. Patients with inflammation—controls	19	7
Diabetes	14	3
Hyperthyroidism	7	2
Arthritis, rheumatism	6	2
I. Endocrine, obesity, growth	7	3
J. Benign brain tumor, neuroma	2	
K. Pregnancies (2)	2	
	115	87
		202

[1] 3 of 5 cachectic.
[2] First 3 of 4 on Stilbestrol (3 prostatic cancers).
[3] Extensive metastases: though patient prepared for surgery, he was terminal.
[4] According to Gruskin's observations, only 3 of the 10 should have given a positive test.

FACTORS THAT AFFECT THE RESULTS OF THE TEST, AND ATTEMPTS TO IMPROVE THE TEST

Since the test proved to have some predictive value, it was considered advisable to try to improve the test and to ascertain the factors that influence it. For lack of space, only some of the more pertinent data and experiments performed are presented, more than one-half of the work actually done being omitted.

Results obtained by diluting serum.—It was thought that instead of adjusting the serum to a constant specific gravity, this aim might be approximated by simply diluting with 0.5 cc. of a 0.85 percent solution of sodium chloride. The 1 cc. of diluted serum was added as described, but in this method the test tubes were only slightly agitated and the readings were made between the 1st and 3d minutes without further dilution. In this modified test the floccules disappeared as a rule within 1 hour.

The incidence of "false positives" in 141 noncancerous students and patient controls fell from 55 percent with the original method to 27 percent in 110 cases with this "dilution method." Unfortunately, the incidence of "false negatives" in known cancer patients increased to 43 percent in 21 cases. Again, the results of the chi-square test indicate that the test revealed a significant difference between the serum from cancer and noncancer patients, or *the test possessed some predictive value but no practical*

value. This was true regardless of whether young healthy subjects or noncancer patients were used as controls.

The effect of diet.—Gruskin has indicated that the presence of the digestive products of dietary protein and fat in the serum are conducive to "false positive" reactions. Twelve young, apparently normal individuals were placed on the 36-hour diet specified by Dr. Gruskin, which is a high-carbohydrate, low-protein, low-fat, and low-caloric diet. Fasting blood samples taken at the end of this period were compared with the fasting blood samples on the same individuals taken after a 12–hour, overnight fast as practiced before obtaining all other blood samples in this study. The same alcoholic extract or solution of "antigen" was employed throughout. The temperature was maintained constant at 20° to 21° C. Five cancer patients were also placed on the 36–hour diet and all gave a 4+ reaction with 0.03 cc. of the alcoholic solution of "antigen." A significant difference between the results on the sera of an overnight fast, with and without the diet, was not obtained.

In addition, 6 noncancer, young, healthy individuals, who had given a "false positive" test, were placed on a strict carbohydrate diet for the evening meal, and a sample of blood was taken before and after breakfast the next morning. The carbohydrate meal the previous evening did not affect the results, though the breakfast containing some egg and milk did. (Definitely lipemic blood is usually positive, regardless of its source.)

These results show that "false positives" are produced by a meal, but precautions other than a 12-hour fast had no significant effect on the incidence of "false positives."

The effect of temperature.—In the early part of this work the tests were performed at room temperature, which was the temperature used by Gruskin. During this time, the room temperature ranged from 20° to 38° C., and it was empirically observed that the sensitivity of the reaction was influenced by temperature.

The effect of temperature on the reaction was studied on a pool of sera from known cancer cases and compared with a pool of sera from healthy individuals. All known factors were kept constant except the temperature at which the test was performed. Glassware and reagents of the test were permitted to reach the desired temperature before performing the tests. The tubes were removed from the water bath only for the 2-minute shaking periods and returned to the designated temperatures as quickly as possible. The temperatures tested in the first experiment were −0.5, 21.5, 37, and 55.5° C. Observations were made at 15-minute intervals for a period of 2.5 hours.

The results are tabulated in table 2. No flocculation was obtained at −0.5° C. with either cancer or normal sera, some differentiation between cancer and normal sera was noted at 21.5° C., with a tendency toward reversibility on standing. At both 37° C. and 55.5° C. heavy flocculations and clouding were obtained in both cancer and normal sera that showed no tendency to reversal over the period of observation. After the

TABLE 2.—*Effect of temperature*

	Time in minutes								
	1	15	45	60	75	90	105	120	150
−0.5° C.									
Cancer pool	Very slightly cloudy	−	−	−	−	−	−	−	−
Normal pool	−	−	−	−	−	−	−	−	−
21.5° C.									
Cancer pool	+++	+++	+++	+++	++	++	+	+	+
Normal pool	+	±	±	± to −	−	−	−	−	−
37° C.									
Cancer pool [1]	++++	++++	++++	++++	++++	++++	++++	++++	++++
Normal pool [1]	++++	++++	++++	++++	++++	++++	++++	++++	++++
55.5° C.									
Cancer pool [2]	++++	++++	++++	++++	++++	++++	++++	++++	++++
Normal pool [2]	++++	++++	++++	++++	++++	++++	++++	++++	++++

[1] Very heavy floccules throughout.
[2] Large heavy floccules throughout.

2-hour period the tests performed at −0.5° C. were warmed to room temperature. The tests performed at the other three temperatures were cooled to −0.5° C. No change in any of the tests was noted by this reversal of temperature, indicating that *the reaction occurs at the time of first contact of antigen and serum.* One may note that in other experiments the tests performed at room temperature and incubated at 4° C., room temperature, and at 37° C. after completion of the test showed a tendency toward reversal of the reaction. With the subjective method of reading, no increase in degree of flocculation has been noted with time or temperature of incubation following the completion of the test.

Tests performed subsequently in a water bath at 20° C. to 22° C. showed a marked decrease in sensitivity. Therefore, the effect of temperature on the test was again evaluated over the range of 10° C. to 39° C. at increments of 5° C. or less. The results are tabulated in table 3. Tests on 2 sera were set up 1 at a time, each in duplicate. No more than 2 were performed at one time, since previous experiments had indicated that a period of longer than 5 minutes of standing after addition of the serum to the "antigen" altered the results. Readings were taken at 1– and 5–minute intervals.

TABLE 3.—*Effect of Temperature*

Temperature	Pool of known cancer sera		Pool of known normal sera	
	1 minute [1]	5 minutes	1 minute	5 minutes
10° C	−, −	−, −	−, −	−, −
15°–16° C	−, −	−, −	−, −	−, −
20° C	+, +	−, −	±, −	−, −
25° C	+++, +++	+++, +++	±, ±	−, −
27° C	+++, +++	+++, +++	+, ±	+, ±
30° C	++++, ++++	++++, ++++	+, +	+, +
31°–31.5° C	++++, ++++	++++, ++++	±, ±	±, ±
33° C	++++, ++++	++++, ++++	++, ++	++, ++
34° C	++++, ++++	++++, ++++	+, +	+, +
37.5–39° C	++++, ++++	++++, ++++	+++, +++	++, ++

[1] Duplicate tests

The differentiation between cancer and normal serum is just apparent at 20° C. This accounts for the decrease in sensitivity of the tests performed at that temperature. The range between 30° and 33° C. yields the most specific results, and the temperature of 31° C. was chosen for subsequent tests.

It seems certain that the specificity of previous results has been affected by room temperature differences and that this factor has to be controlled.

Effect of freezing sera on reaction.—This was studied, since it was the best way to avoid changes from occurring in serum and causing variations in the results of the tests, as a result of standing of the serum at different temperatures and for different durations.

It was found that the results of tests performed on the sera on the day the blood was drawn did not differ significantly from the results on the

UNIVERSITY OF MICHIGAN LIBRARIES

same sera that had been separated from the clot, inactivated at 56° C. for 30 minutes, rapidly frozen in dry carbon dioxide and acetone, and stored at −15° C. for periods up to 3 months. Therefore, all sera were preserved in this manner and brought to room temperature on the day they were tested.

Reproducibility of results of the reaction.—In earlier work we had performed tests on pooled sera, which satisfied us that the results were consistently reproducible. But, we repeated tests on the blood of the same patient sufficiently to indicate that readings on the same patient were reproducible to the extent that under the same conditions of diet and rest the result of the test on the same subject was the same. However, this impression was not statistically valid for the same individual because we had not performed the test many times on a significant number of individuals. As our experience with the test on the sera of different patients increased, and regardless of the statistical predictability of the test when applied to a group of cancer and noncancer patients, the question arose whether we were actually dealing with sera from the same or different populations, especially when the factor of temperature was controlled.

Ten individual tests were then performed on each of 4 known cancer sera and on each of 4 presumably normal individuals. These were performed under identical conditions at 31° C. in a constant-temperature water bath using 0.03 cc. of the same batch of antigen for each test. The results on the 5-minute reading of these tests were then evaluated statistically by assigning a value of 1 to each + in the result, and 0.5 for each ±. Results are given in table 4.

TABLE 4.—*Showing reproducibility of the test on four known cancer sera and four known noncancer sera*

No. of tests	Cancer			Normal			t
	$\bar{x}$	s	$s_{\bar{x}}$	$\bar{x}$	s	$s_{\bar{x}}$	
10	3. 5	±0. 84	±0. 27	0. 95	±1. 09	±0. 34	5. 8
10	3. 8	±. 63	±. 20	. 7	±. 47	±. 15	12. 5
10	3. 7	±. 47	±. 15	1. 0	±. 4	±. 12	13. 5
10	3. 6	±. 69	±. 22	1. 4	±. 65	±. 209	7. 3
40	3. 65	±. 71	±. 11	1. 01	±. 61	±. 096	17. 7

$\bar{x}$=the mean of 10 tests.

s=standard deviation, found by formula $s=\sqrt{\frac{sx^2}{n-1}}$.

$s_{\bar{x}}$=standard error, found by formula $s_{\bar{x}}=\frac{s}{\sqrt{n}}$.

t=significance of the difference, from $t=\frac{\bar{x}}{s_{\bar{x}}}$.

From the value for t, it appears that the hypothesis that the samples are drawn from the same population is invalid. *The evidence again indicates that the test differentiates samples into two populations with different means.*

These and other results were then evaluated by use of the formula $n=t^2c^2$ where $c=\frac{s}{x}$ to determine how many tests would need to be done on each specimen to arrive at a valid evaluation for a single specimen. From the results, it was decided that five individual tests should be done on each serum to determine whether the reading was +, ++, +++, or ++++. That is, at times the serum of a normal person would give a one-plus or occasionally a two-plus flocculation; and, in order to obtain a statistically valid mean, five tests had to be performed. This demonstrated that because we followed Gruskin's directions of calling a one-plus flocculation a positive test some of the "false positives" were not positive. This source of error would not apply to the "false negatives," but would reduce the percentage of false positives to approximately 30 percent. Patients with diabetes, inflammation, and metabolic diseases would still yield a high percentage of "false positives" and render the test impractical.

Effect of standing after layering of serum on alcoholic solution of "antigen."—We were next interested in being able to set up more than one test at a time if possible. Accordingly, an experiment was performed in which the time at which the antigen and serum were permitted to stand undisturbed was varied. This interval had been 3 minutes when the tests were performed singly. Again five tests were done with each interval of time.

The degree of flocculation appeared to increase with lengthening of the time interval with both cancer and normal sera. From the results, it was considered advisable not to do more than two tests at one time, which would still limit the period of standing to 5 minutes.

The role of alcohol in the reaction.—Early in the course of the study and before the importance of temperature control was known and the statistical study of the reproducibility of the tests had been made, results, which need not be cited here, were obtained which indicated that alcohol alone would differentiate between serum from a cancer and noncancer healthy subject, but that the "Gruskin antigen" sensitized the alcoholic coagulation. The results of chemical studies again caused us to suspect that alcohol coagulation alone might be the basis of the reaction.

It should be recalled that in the original test the concentration of alcohol resulting from adding 0.03 cc. of alcoholic solution of "antigen" to 0.5 cc. of serum and their mixing is approximately 6 percent. In the performance of the test, however, the alcohol and serum are permitted to remain in contact for 3 minutes before shaking. And, in this case the concentration of alcohol in contact with the surface of the serum approximates 95 percent for a short period. It is possible that this local concentration of alcohol may cause coagulation of the serum proteins. (It was thought when the preliminary evidence indicated that the phospholipid-rich "antigen" sensitized the coagulation, that the phospholipid by surface action slowed diffusion and prolonged the period of contact of a high concentration of alcohol with the serum proteins.)

To determine the effect of alcohol alone on cancer and normal sera, 5 sera from patients with diagnosed, untreated cancer and 5 sera from apparently normal individuals were tested each 5 times, 1 at a time, with 0.03 cc. of alcohol, at 31° C. Statistical analysis of the results showed that alcohol alone reveals a quantitative difference between cancer and normal sera as judged by the value of *t*. A cancer pool and a normal pool were next tested each 10 times with 0.03 cc. of antigen and also 10 times with 0.03 cc. of absolute alcohol. The results when analyzed statistically failed to show that the antigen sensitized the coagulation caused by the alcohol. Finally an additional study was made to determine whether the action of phospholipids and other substances, which might be present in the antigen, is essential for the reaction. A single batch of "antigen" was divided into 5 aliquots; 4 of the aliquots were treated as follows with the idea of destroying or removing an "antigen" from the alcoholic solution of "antigen":

1. KOH 150° C. 24 hr.—distilled.
2. 150° C. 24 hr.—sealed *in vacuo*.
3. Na fusion distilled.
4. Adsorbed with IR–100, 2 gm. per 10 cc.
   with Zeo Karb, 2 gm. per 10 cc.

A portion of the alcohol from the original alcoholic solution of "antigen" was redistilled and served as a control.

Since the results of these treatments were not significantly different, especially as regards specificity, there is no evidence showing that the "antigen" contributes significantly to the reaction.

Application of the use of alcohol coagulation by a layering technique to the detection of cancer.—Accordingly, the reliability of the use of alcohol alone as a coagulating or precipitating agent for blood protein when placed as a layer over blood serum for the detection of cancer was then studied.

Three types of blood samples were obtained. One from apparently healthy young persons; another from patients entering the dispensary for complaints other than cancer; and a third from patients with untreated diagnosed cancer of any organ. The blood was taken after a 12-hour fast in the morning before breakfast.

Tests were performed 1 at a time, 5 tests on each blood sample, as follows: Only nonhemolyzed, nonicteric, nonlipemic serum is used. All reagents are warmed to 31° C. in a water bath. Pipette 0.03 cc. of alcohol into a Kahn test tube; absolute alcohol is essential. A pipette containing 0.5 cc. of serum is carefully placed in this test tube until the tip of the pipette is approximately 2 mm. above the alcohol and not touching the walls of the tube. One drop of serum at a time is slowly released at 1.5-seconds intervals. Each drop of serum released necessitates a slight raising of the tip of the pipette so that the 2-mm. gap between the alcohol and pipette is maintained. Let stand undisturbed for 3 minutes in water bath at 31° C. Shake for 2 minutes in a Kahn shaking machine. Add 0.5 cc. of 0.85 percent NaCl. Leave for 1 minute in water bath. Shake for 2 minutes. Add 0.5 cc. of 0.85 percent NaCl. Read in 5 minutes and 1

hour at 31° C. Place tubes in refrigerator and read again (18–24 hours).

The results of 5 tests on each sample of serum are presented in table 5. The mean ($\bar{x}$) and the mean ± standard deviation, ($\bar{x} \pm s$) for the 5-minute and 18- to 24-hour reading being given. On the basis of the 5-minute reading, a mean ($\bar{x}$) of 2.5 plus or less for 5 tests on the same sample of serum is "negative" for cancer and above 2.5 is "positive" for cancer. For the 18- to 24-hour readings, a mean of 1.5 or less would be "negative," above 1.5 "positive."

TABLE 5.—*Statistical data on 5–minute and 18- to 24-hour readings*

Type of specimen	Number of specimens	$\bar{x}$	$\bar{x} \pm s$
	5–minute reading		
Cancer	8	3. 5	2. 86 to 4. 14
Normal	15	1. 05	. 49 to 1. 61
Miscellaneous	14	1. 34	. 33 to 3. 01
	18– to 24–hour reading		
Cancer	8	2. 8	1. 69 to 3. 91
Normal	15	. 54	. 26 to . 82
Miscellaneous	14	. 95	. 13 to 1. 96

The number of "positive" and "negative" tests in the 3 groups of subjects is shown in table 6. The 3 patients in the miscellaneous group who gave a "false positive" test had diabetes, as has been observed before.

The results definitely indicate that absolute alcohol is the principal ingredient of the "Gruskin antigen solution." But, they are inadequate to warrant a conclusion to the effect that the "Gruskin antigen" is of no value. This would have to be determined by performing the test on the same sera, using alcohol with and without "antigen." On the basis of our data a large number of sera would have to be tested (each 5 times) in order to obtain a statistically significant difference, if such exists. We do not believe that our data warrant this expenditure of time, especially in view of the lack of a high degree of specificity of the test, a lack which we have not been able to improve.

TABLE 6.—*Results of reactions read at different time intervals*

Time of reading	Results	8 cancer	15 normals	14 miscellaneous
5 minutes	Positive	8	1	[1] 3
	Negative	0	14	11
18 to 24 hours	Positive	7	0	[1] 3
	Negative	1	15	11

[1] These patients had diabetes.

Difficulty in setting up and in reading the test on account of the layering technique.—As the "Gruskin test" is performed, it is difficult to set up any two tests exactly alike because a layering technique—as in some precipitation tests—is used. This is important because a variation between tests in the technique of layering in the alcohol exposes the serum proteins to a variation in the concentration of alcohol with which they are brought into contact. Furthermore, the reading of the test requires considerable experience and is dependent to some extent on the individual reading the test, and small differences cannot be detected. Even an experienced person on repeating the test on the same sera "blindly" will vary from 0 to ++ on the same sera.

When we first started we had the advantage of being trained by Dr. Gruskin, but in some cases even Dr. Gruskin could not satisfactorily differentiate between the reading of a cancerous and a noncancerous serum. *It was chiefly for this reason that the person performing the tests in this study did not know the source of the sera except when pooled sera were used.*

We believe that the two difficulties just referred to account for the inaccuracy of the test, except in cases of diseases, like diabetes, which yield quite uniformly strongly "false positive" tests.

Studies on the alcohol precipitable proteins in the serum of noncancer and cancer-bearing subjects.—It was hypothesized that, if the alcohol in the "Gruskin test" was only acting to precipitate a protein, present in excess or present as a new protein, in the serum, then the tedious and variable method of layering could be discarded, and the alcohol added slowly and directly to the serum (diluted or undiluted) while the serum was being shaken at a controlled temperature. (The precipitate gave a positive Biuret, Hopkins-Cole, and ninhydrin test for protein.)

Various studies were made on all the hypothetical variables in the procedure. Two illustrations will be given of the various procedures used. (A) The serum (1 cc.) was placed in a test tube immersed in a water bath at 25° C. While shaking the test tube, ethyl alcohol in distilled water was added slowly drop-by-drop, so that the final concentration of alcohol would be 17.1, 21.0, 25.6, or 29.9 percent, and the final dilution of the serum or serum-to-alcoholic solution was 1 to 10. After 20 minutes of shaking, the average light transmission of each solution was measured with a Coleman spectrophotometer at 450μ.

(B) Ethyl alcohol (10 cc.) in a series of concentrations in distilled water, ranging from 0 to 60 percent in increments of 5 percent, was placed in a small (25 cc.) stoppered Erlenmeyer flask, which was placed in a water bath at 31.0° C. The serum was diluted 1 to 1 with either distilled water or 0.85-percent NaCl, and also placed in the water bath. After the temperature of the solutions reached 31° C., 1.0 cc. of the diluted serum was added to the alcohol solution in each flask while it was being shaken with a mechanical shaker, all shaking being continued for 1 minute. Reading of light transmission was then made at 1 hour and 24 hours, using the Evelyn colorimeter at a wave length of 600μ. The effect of color

was controlled by standardizing the colorimeter against potassium dichromate solution.

The results with procedure "A" indicated that sera from a cancer patient tended to contain more protein precipitable at a concentration of 25 percent than sera from a noncancer patient, which bore no relation to the protein content of the serum. This difference, however, though uniform, on the average, at a 20-, 25-, and 30-percent concentration of alcohol was not statistically significant.

The sera of 6 noncancer and 6 cancer-bearing subjects when treated by procedure "B" yielded almost identical turbidity values at a concentration of 20, 25, 30, 35, and 40 percent.

Thus, our attempts to avoid the method of layering the alcohol with the serum failed. This indicates that the size and form of the floccules, "amorphous white plates instead of globules or particles" as claimed by Gruskin, is of decisive significance. Shaking breaks and homogenizes the floccules. In other words, the degree of specificity of the Gruskin test does not depend apparently on there being more of an alcohol-precipitable protein in the serum of the cancer-bearing patient, but rather on the physical form that the protein takes at the alcohol-serum interface.

SUMMARY AND CONCLUSION

The "Gruskin test" consists of producing an interface between ethyl alcohol (95%+), containing phospholipid extracted from the embryonic liver of calves (Gruskin "antigen" solution) and the serum of the subject. This results in the formation of floccules that appear as white plates in the presence of cancer. In this entire study we have applied the test to a total of 373 clinically normal persons and non-cancer-bearing patients with various diseases as they would appear in a general practice and to 273 cancer-bearing patients. (These figures include those in table 1 but not those in tables 5 and 6.) The incidence of "*false negatives*" in the cancer-bearing group was 5 percent, even less if certain cases were eliminated as recommended by Dr. Gruskin. The incidence of "*false positives*" was 45 percent. The test clearly and consistently differentiates between pooled sera from cancer- and non-cancer-bearing subjects.

The results have shown that statistically the Gruskin test, as performed by him, is predictive for the presence of known cancer in a person; but the incidence of "false positives" is so high, or the specificity of the test is so low, that it is unreliable for diagnostic purposes.

It has been found that temperature affects the occurrence of flocculation. The reading of the test is so variable that in order to obtain a statistically reliable average reading by the same observer at least five tests must be done on the same serum.

Absolute alcohol not containing the phospholipid ("antigen") extracted from the embryonic liver yielded as good a prediction of the presence or absence of known cancer in the subject as did the use of alcohol containing the phospholipid. The use of absolute alcohol, however, did not

UNIVERSITY OF MICHIGAN LIBRARIES

improve the specificity of the test. Evidence was obtained indicating that the specificity which the test does possess is not due to an increase in the serum of the cancer patient of proteins more readily precipitable by alcohol but rather to the physical form the protein takes at the alcohol-serum interface.

REFERENCES

(*1*) Maver, M. E.: Review of serodiagnostic tests for cancer. J. Nat. Cancer Inst. 4: 571–578 (1944).

(*2*) Davidsohn, I.: Serodiagnosis of malignant tumors. Am. J. Clin. Path. 6: 172-184 (1936).

(*3*) Bing, M., and Marangos, G.: Die diagnostischen Krebsreaktionen. Eine kritische Übersicht. Beitr. z. klin. Chir. 160: 417–444 (1934).

(*4*) Fuchs, H. J.: Neue Beobachtungen zur Diagnostik maligner Tumoren. Verhandl. d. deutsch. Gesellsch, f. inn. Med. Kong. 40: 83–86 (1928).

(*5*) Fuchs, H. J., and Devrient, W. K.: Zur Diagnostik maligner Tumoren aus dem Serum, (CaR). Deutsche med. Wchnschr. 58: 1640–1641 (1932).

(*6*) Wright, W. M., and Wolf, C. G. L.: Serological diagnosis of cancer. J. Cancer Research 14: 370–393 (1930).

(*7*) Kahn, R. L.: Serology in Syphilis Control. Baltimore, Williams & Wilkins Co. (1942).

(*8*) Gruskin, B.: A serum test for the diagnosis of cancer based on a new theory of etiology. Am. J. M. Sc. 177: 476–487 (1929).

(*9*) Kahn, H.: Die Differential diagnose maligner Tumoren aus wenigen Tropfen Blut. Klin. Wchnschr. 3: 920–922 (1924).

(*10*) ———: Über eine einfache Flockungs-Trübungs-reaktion bei malignen Tumoren. Klin. Wchnschr. 2: 1364–1365 (1923).

(*11*) Kopaczewski, W.: Lacto-gélification du sérum humain normal. Compt. rend. Soc. de biol. 116: 869–870 (1934).

(*12*) ———: Les protides sériques dans le cancer. Compt. rend. Acad. d. sc. 201: 1229–1231 (1935).

(*13*) Ascoli, M., and Izar, G.: Die Meiostagminreaktion bei bösartigen Geschwülsten. München Med. Wchnschr. 57: 403–405 (1910)

(*14*) Lavedan, J.: Le séro-diagnostic des tumeurs malignes d'àpres les travaux récents. Paris med. 1: 261–272 (1931).

(*15*) Brdicka, R.: Serologische Untersuchungen mit Hilfe der polarographischen Methode und ihre Bedeutung für die Krebsdiagnostik. Acta, Union internat. contre cancer 3: 13–28 (1938).

(*16*) Roffo, A. H., and DeGiorgi, H.: Die Reaktion des Neutralrot bei Serum Krebskranker und ihre Beziehung zu anderen Farbstoffen. Ztschr. f. Krebsforsch. 25: 136–140 (1927).

(*17*) Sachs, H.: Zur Frage der serologischen Reaktionsfähigkeit bei Geschwulstkrankheiten. Ztschr. f. Krebsforsch. 35: 275–282 (1932).

STUDIES OF VARIOUS TESTS FOR MALIGNANT NEOPLASTIC DISEASES. I. THE REDUCTION OF METHYLENE BLUE BY PLASMA [1] [2]

NILS ERIKSEN, LESTER D. ELLERBROOK, and STUART W. LIPPINCOTT, *Clinical Trials Unit, Cancer Control Branch, National Cancer Institute, Bethesda, Md., and Department of Pathology, School of Medicine, University of Washington, Seattle, Wash.*

INTRODUCTION

Objective: The program of the National Cancer Institute with reference to evaluation of reported tests for the diagnosis of cancer has been recently outlined in an editorial (*1*). Recognition is given in the editorial to the fact that a simple, definitive, diagnostic test capable of distinguishing each type of neoplastic growth early in its development would offer the ideal laboratory aid. However, failing this, a test would still be helpful if it could be used for screening purposes to separate normal individuals from those with cancer with a reasonable degree of accuracy. In the program under way, a number of previously reported tests based upon many different chemical approaches and done under widely varying test conditions have been selected for investigation. The objective is to determine whether any individual test is reliable and applicable as a diagnostic or screening test.

In an attempt to establish the specificity and sensitivity of the tests, they are being applied to normal individuals with a wide age distribution, to patients with various types of histologically proved benign and malignant neoplasms, and to those afflicted with a variety of other types of diseases. All individuals tested are registered in the Washington State Tumor Registry for future follow-up studies, and whenever possible specimens of serum are stored in deep-freeze units so that they may be further studied at any desired time.

A number of other factors involved in the routine performance of the tests and interpretation of individual test results are being investigated to make possible careful evaluations. Among these factors are the reproducibility of results, the possibility of using postprandial or re-

[1] Received for publication September 14, 1950.

[2] Presented in part at the 41st annual meeting of the American Association for Cancer Research, Inc., Atlantic City, April 16, 17, and 18, 1950.

UNIVERSITY OF MICHIGAN LIBRARIES

frigerated specimens, and, in certain cases, the possibility of using different types of specimens (*e.g.*, plasma or serum).

A test meeting the requirements of simplicity, rapidity, and low cost has recently been proposed by Black (*2*). According to Black, the plasma of patients with malignant diseases tended to have a lowered reducing power (increased methylene-blue reduction time) than that of normal individuals. This plasma-methylene-blue reduction test has undergone some changes with succeeding reports, and the source of clinical material and the type of individuals studied have not always been clearly stated; yet abnormal results have been reported in many cases of malignant disease and in a much smaller proportion of cases of other diseases. For these reasons we have made a study of this test in order to determine its value as a practical screening test for malignant disease. The papers of Black and his associates are reviewed in some detail below, and their reported results are summarized in table 1.

TABLE 1.—*Reported results with Black's methylene-blue reduction test*

Literary reference	Percent dye used	Normal		Cancer	
		Number tested	Range of reduction times (minutes)	Number tested	Percent abnormal
(*2*)	0. 10	76	25 percent 9–10; 5 percent 10–11	237	85 percent 10 minutes or over; 80 percent 11 minutes or over.
(*4*)	. 13	------	9–10.5 doubtful--------	136	32 percent 9–10.5 minutes; 45 percent 11 minutes or over.
(*5*)	. 15	[1]	----------	[2] 1, 000	75–80 percent.
(*6*)	. 15	1, 366	0.01 percent over 10----	468	
(*7*)	. 15±	134	98 percent 8 minutes ± 2 S. D. (6.5–9.5)	82	52 percent over 9.5 minutes.

[1] Almost 2,000 controls.
[2] This is apparently an approximate figure.

REVIEW OF PAPERS ON BLACK'S PLASMA-METHYLENE-BLUE REDUCTION TEST

In his first paper Black (*2*) stated that the plasma or serum of patients with cancer usually has a prolonged methylene-blue reduction time when measured by the technique outlined by him. The procedure given was as follows: 0.2 ml. of a 0.1-percent solution of methylene blue was mixed with 1 ml. of plasma or serum (fasting or postprandial) in a Kahn tube. The tube was then immersed in boiling water, and the time required for complete decolorization of the dye was noted. The reducing times of normal individuals showed the "greatest concentration of values below 9 minutes." In a group of 76 normal individuals the percentages of plasma samples giving various reduction times were stated to be as follows: 8 percent, 6-7 minutes; 21 percent, 7-8 minutes; 41 percent, 8-9 minutes; 25 percent, 9-10 minutes; 4 percent, 10-10.5 minutes; 1 percent, 10.5-11 minutes. The plasma of 138 patients with nonmalignant disease tended to give values below 10 minutes, but the majority of 237 cases with malig-

nant neoplastic diseases gave reduction times of 10 minutes or more. It appears from his distribution diagram (Figure 2, reference 2) that about 85 percent of these values were 10 minutes or over, 80 percent were 11 minutes or over, and about 62 percent were 12 minutes or over.

Black also made use of a 0.10-percent solution of brilliant cresyl blue. One ml. of plasma and 0.2 ml. of this solution were mixed and then heated in boiling water for 10 minutes. Complete reduction to a grey-white color was obtained in the nonmalignant groups, while incomplete reduction was often found with patients with malignant neoplastic disease. The final evaluation of the reducing power of the blood rested upon the reaction with both dyes. Although the normal and "cancer" methylene-blue values showed some overlap, it was considered possible in most cases to make correct differentiation by virtue of the differences in reaction of the brilliant cresyl blue.

In a later paper Black (*3*) aimed to describe a standard method of calibration of various lots of methylene blue, so that more uniform results would be possible in the plasma-reducing test. The procedure was based upon the finding that cysteine also reduces methylene blue when heated in boiling water, presumably because of its sulfhydryl group. He stated that the results indicated a "linear relationship between the cysteine concentration and the reducing power, and a definite limiting value of cysteine concentration for reduction of methylene blue." Actually his results (Figures 1 and 2, reference 3) do not show a linear relationship between the cysteine concentration and the reduction time. Tests of cysteine solutions of different concentrations indicated that for a particular concentration of cysteine there was "no appreciable difference in the reducing time of methylene blue on varying [its] concentrations between 0.1 and 0.2 percent although 0.08 percent shows a decided difference." Similar findings were obtained when a plasma sample was used to reduce the methylene-blue solutions; the reduction time for 0.08-percent dye was 5.5 minutes and that for both 0.13- and 0.20-percent dye was 8 minutes. In view of the parallel results obtained with cysteine hydrochloride and plasma, Black stated that the cysteine solutions may be used to test the reactivity of different lots of methylene blue. Just how this was to be done was not made clear. Moreover, he did not explain why a specimen of plasma gave identical reduction times with 0.13- and 0.20-percent dye, while in his previous paper he reported that a control plasma sample gave a reduction time of 9 minutes with a 0.10-percent dye solution and one of 15 minutes with a 0.12-percent solution.

In a later paper by Black, Kleiner, and Bolker (*4*) describing a heat-coagulation reaction of plasma, it was stated, "As reported in the papers already referred to [the two mentioned above], a methylene-blue reducing time of 11 minutes or more is considered to be suggestive of the presence of malignancy; while values of 8.5 minutes or less are considered normal. When values between 9.0 and 10.5 minutes are obtained, the plasma is also tested with brilliant cresyl blue. Incomplete reduction of this dye is interpreted as suggestive of cancer, whereas complete reduction is con-

sidered to indicate a normal type of reaction." When 136 cancer patients were tested by this procedure, 23 percent gave methylene-blue reduction times of 6 to 8.5 minutes, 32 percent gave reduction times of 9 to 10.5 minutes, and 45 percent gave reduction times of 11 to 16 minutes. Abnormal reactions were obtained by either or both the reducing-power studies or the heat-coagulation value in 86 percent of 194 cases of cancer. Of 1,539 cases tested by the reducing-time studies alone, 80 percent of 533 cancer cases were identified.

In 1948 Black (*5*) reported the use of the methylene-blue reduction test upon almost 2,000 control individuals, 1,000 diverse cases of nonneoplastic diseases and 1,000 cases of cancer. Distinctive prolongations of the reduction times were noted in 75 to 80 percent of the cancer cases, while these prolongations were not found in nonneoplastic diseases, with the exception of cases of pregnancy, tuberculosis, rheumatic fever, and cirrhosis. He also stated that the elevated reducing times in cancer cases are readily reversible after adequate therapeutic procedures *via* surgical resection or radiation. The procedure given involves the use of a 0.15-percent solution of methylene blue instead of the 0.10- or 0.13-percent solutions used previously. The tests were performed in Wassermann tubes instead of the Kahn tubes used previously.

In 1949 Black (*6*) again reported the use of a 0.15-percent solution of methylene blue. The studies had been extended to include the testing of 1,366 presumably healthy individuals, and only 0.01 percent of control individuals tested gave reduction times above 10.0 minutes. (Most of the control individuals were actually cancer-detection-center patients.) In view of this he considered times of 10½ minutes or longer as abnormal. When either the reducing time or the heat-coagulation values were abnormal, the reaction was considered to be abnormal or consistent with the presence of malignancy. Using these criteria, positive tests were obtained in 416 of 468 cases—an identification accuracy of 89 percent. Approximately one-half of these cases were ambulatory and pursuing normal activity, so it was concluded that these reactions are not based upon cachexia or a long-standing debilitating process. The reduction technique alone gave no more than 60 to 70 percent positive tests. Normal values were obtained by both techniques with 99 percent of 977 healthy control individuals; with 91 percent of 588 patients with nonneoplastic diseases other than cirrhosis, pregnancy, acute rheumatic fever, and tuberculosis; and with 92 percent of 116 cases of nonmalignant neoplasms. Drug-free plasma specimens were specified because it was found that streptomycin, penicillin, and pyribenzamine gave elevated results in both tests. It was also stated that the average individual tends to have about the same reduction time over a number of years; a cancer patient does not give higher and higher values as the tumor enlarges but, rather, he will have a particular reducing time that will remain the same unless successful therapy causes it to return to normal.

Kleiner, with Stettner and others (*7*), has recently reported on the use of the methylene-blue reduction test both alone and in conjunction with

the heat turbidity index. They stated that the plasma should be separated from the cells within 1 hour, that the tests should be performed as soon thereafter as possible, and that the blood should be stored in the refrigerator if it is not to be tested immediately. They warn that many drugs interfere with the test.

In their "present procedure" 1 ml. of fasting plasma is placed in a 10- by 100-mm. Wassermann tube, and to this is added exactly 0.2 ml. of a standardized solution of methylene blue in distilled water containing approximately 150 mg. of dye per 100 ml. of solution. All patients designated as normal had been examined by a physician and passed clinically as normal at about the time the blood was withdrawn for testing. The majority of these normal subjects came from a cancer-detection clinic. The other patients were referred from clinics, outpatient departments and wards of several hospitals. No case was included in the malignant series until the diagnosis had been confirmed by histologic findings.

The mean reducing time in 134 normal persons was 8 minutes, with a standard deviation of ¾ minute. Ninety-eight percent of the values fell within a range of plus or minus two standard deviations from the mean. Consequently a reduction time of 10 minutes or more was considered to be abnormal. The reduction times obtained in 82 cases of malignant neoplasms ranged from 6½ to 14½ minutes, and 52 percent of them were 10 minutes or more. Most (about 92 percent) of the times obtained in 239 patients with a variety of nonmalignant diseases were in the normal range, with the exception of some of those obtained in pregnancy, pulmonary tuberculosis, acute rheumatic fever, or quantitative disturbances of the blood proteins. The group with known plasma-protein abnormalities was excluded from consideration, since the test results "should obviously be corrected for the concentration of protein present."

The heat-turbidity index was abnormal in 80 percent of the cases of malignant disease, and in 96 percent of these cases at least one of the two tests gave abnormal results. Of the cases having at least one abnormal test result practically all of the patients were ill, and the majority had some form of malignant disease. Since only two patients with normal combined tests had cancer, the authors conclude that if both test results are within normal limits malignant disease is almost certainly absent; on the contrary, if either test performed on a drug-free specimen gives an abnormal result, and if tuberculosis, pregnancy, acute rheumatic fever, and quantitative blood-protein abnormalities are ruled out, the probability is that cancer is present.

MATERIALS AND METHODS

Selection of Subjects for Testing

Normal controls.—Blood specimens were collected from a group of 145 normal individuals ranging in age from 18 to 66 years. One hundred eighteen of these specimens were obtained after an overnight fast, and 27

were taken 2 to 4 hours after breakfast. These subjects were all healthy, as far as could be determined by history, and in nearly all cases by X-ray examination of the chest, temperature, white- and red-blood-cell counts, and blood-hemoglobin concentration. None of these individuals were taken from what may be considered two objectionable sources, a hospital out-patient department and a cancer-detection center.

Patients with neoplastic diseases.—The patients with neoplastic diseases included 35 with benign and 144 with malignant lesions. All individuals were tested shortly after hospital admission and prior to any form of therapy, terminal cases and diseases of long duration thus having been practically excluded. No patient had received a transfusion within 1 week prior to testing. Blood specimens for the test were obtained following overnight fasting. All diagnoses were established by microscopic examination of tissues removed at biopsy, operation, or autopsy. An adequate selection was made of many types of neoplasms and classification was based on histogenesis. Careful review of the clinical and laboratory records of each individual with malignant disease indicated that 80 already had proved metastases, whereas in the other cases metastases were not recognized at the time of testing.

Patients with miscellaneous diseases.—There were 167 patients with miscellaneous diseases. A large number of these patients were selected because they had diseases that might be confused clinically with cancer. These individuals were usually selected prior to operation when at that time it could not be established whether or not the patient might have a cancer; *e. g.*, differential diagnosis between peptic ulcer and gastric carcinoma. A number of patients with a variety of other diseases were tested, not with the assumption that there might be confusion between these diseases and cancer clinically, but on the basis that test results might give values within the cancer range. This method of choosing patients helped us to study the specificity of the test as well as its possible practicability in a screening program.

Technique of test

A specimen of venous blood was drawn into a dry syringe and transferred to a tube containing approximately 20 mg. of potassium oxalate per 10 ml. of blood. The oxalate and blood were mixed by gentle inversion of the cork-stoppered tube. Within 1 to 3 hours the plasma was separated from the cells by centrifuging for 20 minutes at 1,100 r. p. m. in an International No. 2 Centrifuge fitted with a 16-place head.

The tests were carried out according to the following procedure, which in all essential details is Black's (*6*) most recently published method: (*a*) 1 ml. of plasma was delivered from a volumetric pipette into a 13- by 100-mm. Pyrex tube; (*b*) 0.20 ml. of 0.15 percent methylene-blue solution was added from a 0.2-ml. serological pipette, and the materials were mixed thoroughly by rotation of the tubes; (*c*) the tube was suspended to a depth of about 3 inches in a vigorously boiling water bath, and the time

required for the last visible trace of blue color to disappear was measured to the nearest ½ minute.

We found it convenient to suspend the tube by means of a piece of rubber tubing that slipped easily over the open end of the tube and was held by means of a clamp, allowing the tube to be lowered as the water level became lower because of evaporation and at the same time preventing water from splashing into the open end of the tube. A 600-ml. beaker served as a water bath.

The methylene blue used in Black's first study (*2*) was made by Mallinckrodt and differences in reaction were found with several other samples tested. For this reason the methylene-blue solutions used throughout this work were prepared from a single bottle of methylene-blue powder (Mallinckrodt, U. S. P. XII). All routine tests were run with a solution of 0.15 percent strength, made by weighing out 0.150 gm. of the powder and diluting to 100 ml. with distilled water in a volumetric flask. The solutions were transferred to glass-stoppered Pyrex bottles, and stored in a refrigerator when not in use. The solutions were thoroughly mixed before each use.

PROBLEMS OF THE TECHNIQUE

Reproducibility

The conditions chosen by Black for his test result in a reaction that takes place, after the first minute or two of heating, in a semi-solid medium, broken up in most cases by the evolution of water vapor and dissolved gases. The coagulation of the plasma proteins prevents free intermixture of the reactants. Consequently islands of unreduced methylene blue remain after the main body of the coagulum has been decolorized. The reaction is timed until the last of these islands disappears. So much would seem to depend on the physical nature of the clot formed that it was deemed advisable to make a thorough study of the reproducibility of results.

The error of measurement attached to the determination of a single reduction time was estimated by comparing the results of duplicate determinations on 63 plasma specimens having reduction times ranging from 6½ to 22 minutes. The variance for each pair of measurements was taken to be ½ the square of the difference between the 2 measurements. The average variance was found to be 0.43 giving a standard error of a single measurement of 0.66 minute, the square root of the average variance. The ratio of this standard error of measurement to the average reduction time, the coefficient of variation of measurement, is then 6.1 percent.

More reliable estimates of the error variance for an individual specimen were computed from the results of 10 replicate determinations done with a normal-plasma specimen and similarly with an abnormal specimen. For the normal specimen, having a mean reduction time of 7.2 minutes, the variance, standard deviation, and coefficient of variation were found to be 0.28, 0.53, and 7.4 percent, respectively. For the abnormal speci-

men, having a mean reduction time of 16.1 minutes, the corresponding figures were 0.97, 0.98, and 6.1 percent, respectively.

The combined results for the 65 plasma specimens show a coefficient of variation of about 6½ percent. These specimens being assumed to be a representative sample, the coefficient of variation indicates that there is a probability of 0.68 that any single determination of the reduction time will be within 6½ percent of the true value.

Standardization of the Reagent

A satisfactory method of standardizing the methylene-blue solution used for the reduction test has yet to be proposed. Originally Black (*2*) tested new solutions of methylene blue with a control plasma. If the reduction time was different from that obtained with the previous solution, he diluted or concentrated the new solution, as the case required. How the control plasma was preserved, or reproduced at the desired time, was not made clear. In a subsequent paper (*3*), Black suggested the use of cysteine hydrochloride to standardize methylene-blue solutions. He stated explicitly, "The reaction between cysteine and methylene blue shows a linear relationship, which may be used for calibration of different lots of methylene blue." Yet in the same paper he also stated that there is no appreciable difference in the time of reduction of methylene blue by cysteine hydrochloride on varying the concentration of the dye between 0.1 and 0.2 percent, which is precisely the range wherein a reliable indicator of variations in concentration would be necessary for standardizing a 0.15-percent solution. Somewhat later Black (*8*) recommended calibrated solutions of 0.15-percent methylene blue prepared by Endo Products, Incorporated. These solutions are said to be standardized against plasma from human subjects and also by a spectrophotometric method, the details of which are not explained in the commercial literature describing the product. Stettner and associates (*7*) defined a primary methylene-blue standard as a solution (containing approximately 150 mg. of dye per 100 ml. of solution) that gives a reduction time of between 7 and 9 minutes for normal plasmas.

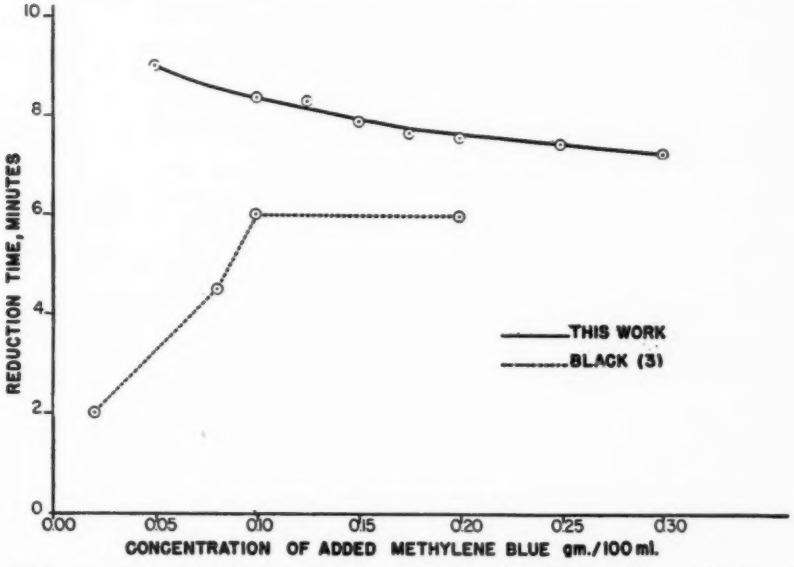
The test results presented in this paper were obtained by the use of 6 different methylene-blue solutions, numbered consecutively from 1 to 6 for convenient reference here. It was noted that these supposedly identical solutions, prepared at different times from the same bottle of methylene-blue powder, did not all behave identically. For example, 2 of the solutions were tested on one occasion against 6 specimens of plasma, and were also compared in a Coleman Junior spectrophotometer at $\lambda =$ 660 mμ, the absorption maximum of methylene blue. The results are shown in table 2. According to reduction times, solution 5 would be considered the more concentrated of the two; optically, however, solution 3 appears to be slightly more concentrated than the other. Similar contradictions were observed on other occasions with other solutions. We are unable to account for these differences, but it is obvious that two

solutions could not be considered identical upon the basis of optical agreement alone.

TABLE 2.—*Comparison of plasma reduction times with optical densities of methylene-blue solutions*

Plasma specimen No.	Reduction time (minutes)	
	Solution 3	Solution 5
5443	7½	9½
5446	7½	9
5447	7½	9½
5449	9	10
5469	8	9½
5470	8	8½
Average	7. 9	9. 3
	Optical density of 1:250 dilution at λ=660 mμ (arbitrary units)	
	0. 780	0. 745

The relation between concentration of methylene blue and time of reduction by cysteine hydrochloride is shown graphically in text-figure 1.



TEXT-FIGURE 1.—Relation between concentration of methylene blue and time of reduction by 0.0325 M cysteine hydrochloride at 100°C.

The method used was the same as that described for plasma, except that 1 ml. of 0.0325 M cysteine hydrochloride was substituted for the plasma. The concentration of methylene blue was varied, but the volume of solution added was always 0.2 ml. A curve constructed from Black's

data (*3*) is shown in the same text-figure. The two sets of data are seen to be in entire disagreement. More important than this difference is the fact that neither curve would be suitable for accurately standardizing methylene blue in the concentration interval 0.1–0.2 percent. The inverse relation between concentration and time, confirmed by us on several occasions, suggests variations in the reaction kinetics with changing methylene-blue concentration.

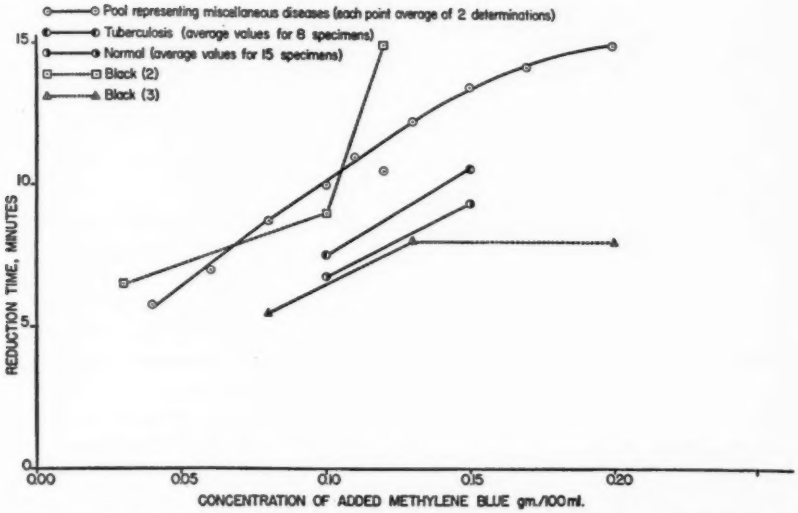
Because of the lack of a suitable chemical or physical method of standardization, we compared successively prepared methylene-blue solutions on the basis of reduction times obtained simultaneously with duplicate samples of a series of 10 to 20 plasma specimens having a wide range of reduction times. With consideration for the probable error attached to a single determination, the observed differences between duplicate samples were analyzed by statistical methods. In addition, an indirect check between solutions was made by comparing distributions of normal values obtained with each solution. If two or more solutions were found to give reduction times that were statistically indistinguishable, the solutions were considered to be identical, and test results obtained by their use were not differentiated according to the solution used. If a solution was found to give reduction times that were significantly different from those given by other solutions, test results obtained by its use were treated separately; *i. e.*, evaluated by comparison with normal-plasma reduction times given by the same solution. For the reasons just set forth, solutions 1 and 2 were combined into one group, solution 3 was considered by itself, and solutions 4, 5, and 6 were combined into another group.

Relation Between Concentration of Methylene Blue and Time of Reduction by Plasma

In his series of papers dealing with the methylene-blue test, Black has reported test results based on methylene-blue solutions having concentrations of 0.10, 0.13, and finally 0.15 percent (*2-6*). The adoption of the higher concentrations apparently was a result of his observation that between methylene-blue concentrations of 0.10 and 0.20 percent the reducing times obtained with plasma did not differ appreciably. If this observation were correct, there would be no need for accurate standardization of methylene-blue solutions, provided they were within the specified range of concentration, yet Black has repeatedly mentioned the importance of standardizing each freshly prepared solution.

In our laboratory a steady increase in reduction time with increasing methylene-blue concentration has invariably been observed with plasma. Representative data are shown in text-figure 2. Two sets of Black's data are included for comparison. It is seen that his curves not only disagree with ours but also seemingly contradict each other.

The effect of any possible small errors in the preparation of 0.15-percent-dye solution was investigated by testing 10 plasma samples simultaneously with 0.14, 0.15, and 0.16-percent solutions prepared by dilution of a



TEXT-FIGURE 2.—Relation between concentration of methylene blue and time of reduction by plasma.

single 0.3-percent solution. The reduction times ranged from 8 to 13½ minutes, and in each case an increase with increasing methylene-blue concentration was noted. Mean increases in reduction time of 4.3 percent between 0.14 and 0.15-percent methylene blue, and of 5.6 percent between 0.15 and 0.16-percent methylene blue, were observed. The relationship between concentration and reduction time is thus approximately linear in the concentration interval 0.14 to 0.16-percent methylene blue. An error of 0.01 percent (absolute) in the preparation of a methylene-blue solution would cause reduction-time errors of the same order of magnitude as the coefficient of variation (6½ percent) determined for the test-technique itself.

EFFECT OF TUBE-SIZE ON REDUCTION TIME

Originally Black (*2*) carried out his test in Kahn tubes, the exact dimensions of which he did not specify. Later he switched to Wassermann tubes (*3-5*), again of unspecified dimensions. Stettner *et al.* (*7*) specified Wassermann tubes measuring 10 by 100 mm. Throughout our work 13 by 100-mm. Pyrex tubes with an inside diameter of approximately 11 mm. were used. The effect of tube-size on the reduction time was estimated by comparing results obtained by the use of these tubes with results obtained by the use of tubes measuring 12 by 100 and 9 by 75 mm. In a series of 10 comparisons involving the 2 larger tubes, no greater differences were found than would have been expected for duplicate determinations in 13-mm. tubes. In a series of 11 comparisons between the 13 by 100 and the 9 by 75-mm. tubes, the mean value of reduction times determined in the smaller tubes did not differ significantly from that

of reduction times determined in the larger tubes, but the average variance between results for duplicate specimens was greater than would have been expected for duplicate specimens tested in 13-mm. tubes. It appears then that the tube-size may affect the reduction time, but that differences caused by small variations in size are negligible.

The rubber sleeves routinely used to suspend the tubes in the water bath effectively increased their length but duplicate tests of 22 specimens indicated that the results obtained with and without the use of the sleeves were not significantly different.

FACTORS INVOLVED IN APPLICATION OF THE TECHNIQUE

Plasma *Versus* Serum

The difference between the reduction times of plasma and serum obtained from the same whole-blood specimen was measured in 21 cases. The mean difference (0.29 minute) between the plasma and serum values did not differ significantly from zero at the 5-percent level. The average coefficient of variation for the 21 plasma-serum pairs was 5.7 percent, which is almost identical with the average coefficient of variation computed for duplicate determinations with plasma. We therefore concur with Black (*2*) that serum may be used in place of plasma, although all of our work was done with plasma.

Fasting *Versus* Postprandial Subjects

In his first publication on the methylene blue test, Black (*2*) stated that it is not necessary to limit the test to blood specimens taken from individuals in a fasting state. Since it would be advantageous to have no restrictions concerning the absorptive state of the individual whose plasma is to be tested, an attempt was made to measure the effect that eating might have on the reduction time.

Twenty-five pairs of plasma specimens were obtained from normal individuals before, and approximately 2 hours after, breakfast. Twenty-nine such fasting-postprandial pairs were also obtained from cancer patients. A statistical analysis of the reduction times of these specimens showed that a series of postprandial values may be expected to have the same distribution as a corresponding series of fasting values. Although the average variance between members of a pair was found to be slightly greater than the error variance of a single specimen (significantly greater at the 5-percent level, in the case of the cancer specimens), this increased variance might just as well be attributed to the actual differences between successive specimens as to the effect of the intervening meal; therefore it cannot be said with confidence that a fasting value is more reliable than a postprandial value. For the sake of uniformity, only fasting values are reported in this paper, with the exception of 27 values that were used to help establish a normal distribution. It has been shown that for this purpose groups of fasting and postprandial values may be used interchangeably.

Fresh *Versus* Refrigerated Specimens

Both Black (*6*) and Stettner *et al.* (*7*) have implied that the results of the methylene-blue reduction test are not affected by storage of the plasma in a refrigerator for 24 hours or less. Because of the practical advantage of being able to store the specimen before use, we considered this point worthy of verification.

Certain plasma specimens were tested both as soon as possible after the blood was taken and after refrigeration for 24 hours. In a total of 76 such comparisons a mean decrease in reduction times of 0.8 minute was observed. This difference was found to be significantly different from zero at the 1-percent level. Eighteen specimens were also tested after an additional day of refrigeration. The mean decrease over the 2-day storage period was 1.8 minutes. This difference is also significantly different from zero at the 1-percent level.

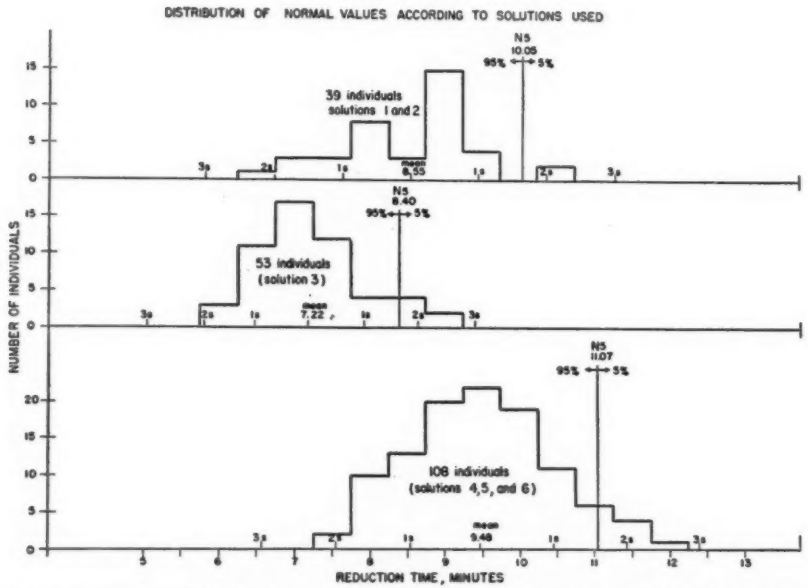
Our results indicate that there is a definite tendency for the reducing time to decrease during storage, and that a significant decrease is very likely to occur during the first 24 hours of refrigeration. For this reason all of our tests were run on fresh specimens of plasma.

SENSITIVITY AND SPECIFICITY OF THE TEST

The evaluation of the sensitivity and specificity of the test rests upon a comparison of reduction times obtained in cancer and other diseases with the appropriate normal reduction times. The distributions of normal reduction times obtained with the 3 groups of methylene-blue solutions used in this study are shown in text-figure 3. Seven of the individuals included in the first group and 48 of those included in the second group are also included in the last group. A net total of 145 individuals are thus represented in the total of 200 normal values.

The selection of points differentiating between normal and abnormal values was based on statistical considerations presented by Dunn and Greenhouse *(9)*, whereby the highest 5 percent of normal values are arbitrarily designated as false-positive test results. A critical reduction time, N_5, setting off the highest 5 percent of the values from the other 95 percent, was computed for each group of normal values from the formula $N_5=m+1.645s$, where m is the mean of the group of normal values and s is the standard deviation. This computed value of N_5 is the best estimate of the true value, a Gaussian, or "normal," distribution being assumed. There is a probability of approximately 95 percent that the true value lies in the interval defined by the expression $N_5 \pm 2\left(\frac{1.534s}{\sqrt{n}}\right)$ where n is the number of individuals in the group under consideration. The computed values of N_5 and the approximate 95-percent confidence intervals for the 3 distributions of normal values are as follows:

Solutions 1 and 2	N_5=10.05 minutes (9.60–10.50).
Solution 3	N_5= 8.40 minutes (8.10–8.70).
Solutions 4, 5, and 6	N_5=11.07 minutes (10.78–11.36).



TEXT-FIGURE 3.—Distributions of normal values according to methylene-blue solutions used.

Any reduction time exceeding the appropriate computed value of N_5 was taken to be a positive result. Since the times were measured only to the nearest ½ minute, the lowest experimentally obtained positive results corresponding to each of the 3 groups of solutions were 10½, 8½, and 11½ minutes, respectively.

The 108 normal individuals tested with solutions 4, 5, and 6 were from 18 to 66 years of age, with a median age of approximately 28. The mean reduction time for the 18–28 age group was 9.46 minutes; for the 29-66 age group, it was 9.50 minutes. The difference between these means is not significantly different from zero at the 5-percent level. It is concluded that the age of the subject tested did not markedly affect the test results, and that it probably is not necessary to have a normal control group with an age distribution identical to that of the group of cancer patients.

The results obtained in 179 cases of neoplastic diseases prior to treatment are shown in table 3. In the 144 cases of malignant neoplasms studied, there were 69 positive test results, or 48 percent, which is significantly greater than the 23-percent positive results in the group of benign neoplasms. In the various groups of malignant neoplasms, the percentages of positive results ranged from 38 to 53. These were found not to differ significantly among themselves, assuming the true rate to be 0.48. Therefore, the best estimate in any category is the over-all rate of 0.48. In those cases with known metastases, 50 percent of the results were positive; where there were no known metastases, 45 percent of the results

were positive. However, these percentages do not differ significantly. Individual test results and confirmed diagnoses for all patients with neoplastic diseases are listed in tables 6 and 7 (see Appendix).

TABLE 3.—*Summary of results obtained in 179 cases of neoplastic diseases prior to treatment*

	Number cases	Number positive	Percent positive
Benign	35	8	23
Malignant:			
Gastrointestinal	38	20	53
Breast	15	6	40
Bronchiogenic	16	6	38
Prostate	15	7	47
Carcinoma, type undetermined	9	4	44
All other types	51	26	51
Total	144	69	48
Known metastases	80	40	50
No known metastases	64	29	45

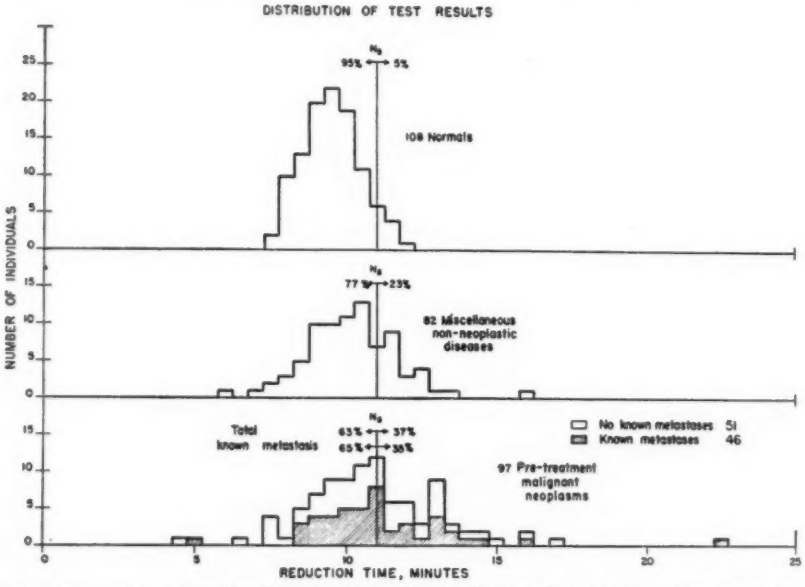
The results obtained in 167 cases of miscellaneous nonneoplastic diseases are shown in table 4. The over-all percentage of positive results was 26. The frequency of positive results in cases of hyperplasia was comparable to that observed in malignant processes and somewhat less in cases of gastrointestinal lesions. In other groups of pathologic processes, the percentages of positive results ranged from 7 to 34. These were found not to differ significantly among themselves, assuming the true rate to be 0.26. Therefore, the best estimate in any category is the over-all rate of 0.26. Individual test results and diagnoses for all patients with nonneoplastic diseases are listed in table 8 (see Appendix).

TABLE 4.—*Summary of results obtained in miscellaneous nonneoplastic diseases*

	Number cases	Number positive	Percent positive
Lesions that might clinically be confused with cancer:			
Hyperplasia	23	9	39
Gastrointestinal lesions	16	5	31
Inflammation (chiefly chronic)	20	4	20
Other pathologic processes:			
Tuberculosis and syphilis [1]	21	3	14
Circulatory diseases	22	6	27
Metabolic disorders	13	3	23
Trauma [2]	14	1	7
Others [2]	38	13	34
Total:	167	44	26

[1] In some of the cases of tuberculosis streptomycin was being given, and in all cases of syphilis penicillin was being given.
[2] Some of these patients may have received drug therapy.

A direct graphic comparison of the results obtained with solutions 4, 5, and 6, excluding results for benign neoplasms, is shown in text-figure 4, which represents a large portion of our experimental results. The overlap between the groups of normal and diseased individuals is apparent.



TEXT-FIGURE 4.—Distribution of test results obtained with methylene-blue solutions 4, 5, and 6.

For relatively large samples of a given population, the number of results that would be expected to exceed the computed value of N_5 by any amount, however small, may be calculated by a statistical method. The prediction is based on the Gaussian distribution of test results having the same mean and standard deviation as the experimental results for the sample actually selected. The area under the theoretical distribution curve represents the total number of cases under consideration, and the portion of the area beyond the N_5 point represents the expected fraction of positive results. Because the experimentally determined reduction times are measured only to the nearest ½ minute, the predicted percentage of positive results will tend to exceed the counted percentage by an amount depending on the time interval between the N_5 value and the next higher ½ minute, provided that the distribution of experimental results is symmetrical, or nearly so.

Predicted values are compared with our experimental results in table 5. In order to calculate the predicted values, it was necessary to consider

TABLE 5.—*Comparison of experimentally obtained and predicted percentages of positive results*

	Number cases	Number positive		Percent positive	
		Counted	Predicted	Counted	Predicted
Malignant neoplasms	144	69	79	48	55
Miscellaneous diseases	167	44	56	26	34

separately the results obtained with each group of solutions. The predictions based on each group were then combined, having been appropriately weighted according to the number of results represented in each group.

SUMMARY

The methylene-blue reduction test of Black has been applied to plasma specimens obtained from normal individuals and individuals having various neoplastic and nonneoplastic diseases. One hundred forty-five normal individuals, ranging in age from 18 to 66 years, were tested. The distribution of reduction times for the younger half of this group did not differ significantly from the distribution of values for the older half. A reduction time equal to, or exceeding, any one of the highest 5 percent of the normal values was considered to be abnormal. One hundred forty-four individuals with malignant neoplasms and 35 individuals with benign neoplasms were tested before they had received any form of treatment, so far as it was possible to determine. Abnormal reduction times were obtained in 48 percent of the group with malignant neoplasms and in 23 percent of the group with benign neoplasms. Eighty of the individuals with malignant neoplasms were known to have metastases; of these, 50 percent gave abnormal results. Of 167 individuals with miscellaneous nonneoplastic diseases, 26 percent gave abnormal results.

The standard error of a single measurement was found to be approximately 6½ percent of the estimated true value of the reduction time.

Differences between supposedly identical solutions of methylene blue prepared from the same lot of powdered reagent were noted, and these differences were taken into account in evaluating test results.

No significant difference was observed between reduction times given by plasma and serum obtained from the same whole-blood specimen.

A slight but significant decrease in reduction time was observed on the average in plasma specimens stored overnight in a refrigerator.

No significant difference in reduction times was observed between plasma specimens obtained before, and approximately 2 hours after, breakfast from normal individuals. Apparently significant differences between plasma specimens obtained from cancer patients before and after breakfast were observed in several instances.

CONCLUSION

The results obtained in this study are in agreement with other reports that the plasma methylene-blue reduction technique is not a specific diagnostic test for malignant neoplastic diseases. Since only approximately 50 percent of the specimens obtained from patients with malignant neoplastic diseases gave abnormal results, the test does not appear to be suitable for screening purposes.

ACKNOWLEDGMENT

The statistical analyses of data reported in this paper were carried out under the supervision of Mr. S. W. Greenhouse, Biometrics Section, National Cancer Institute.

929548—51——8

REFERENCES

(1) LIPPINCOTT, S. W., and DUNN, J. E., Jr.: Studies of various tests for malignant neoplastic diseases. An editorial. Am. J. Clin. Path. 20:655–656 (1950)

(2) BLACK, M. M.: Changes in the reducing power of serum or plasma of patients with malignant neoplastic disease. Cancer Research 7: 321-325 (1947).

(3) ———: Sulfhydryl reduction of methylene blue with reference to alterations in malignant neoplastic disease. Cancer Research 7: 592–594 (1947).

(4) BLACK, M. M., KLEINER, I. S., and BOLKER, H.: Changes in the heat coagulation of plasma from cancer patients. Cancer Research 8: 79–82 (1948).

(5) BLACK, M. M.: Plasma reduction of methylene blue. Science 108: 540–541 (1948).

(6) ———: Proceedings of the conference on cancer detection. American Cancer Society, Inc., Portsmouth, N. H., 1949, pp. 138–139, 150–151.

(7) STETTNER, M. M., BARON, H., LOWENSTEIN, B. E., GREENWALD, A. E., MERSHEIMER, W. L., and KLEINER, I. S: A clinical evaluation of the Black-Kleiner-Bolker blood test for malignant disease. J. Lab. Clin. Med. 35: 136–140 (1950).

(8) BLACK, M. M.: Personal communication, August 1948.

(9) DUNN, J. E., and GREENHOUSE, S. W.: Principles and criteria for the development and evaluation of cancer diagnostic tests. Federal Security Agency, Public Health Service, Publication No. 9, 1950.

APPENDIX

TABLE 6.—*Patients with malignant neoplastic diseases*

No.	Diagnosis	Age	Sex	Results in minutes according to solutions used		
				1, 2	3	4, 5, 6
1	Basal cell carcinoma, rectum	64	M	------	------	8
2	Squamous cell carcinoma, skin	92	M	------	------	9
3	Squamous cell carcinoma, face	77	F	------	------	10½
4	Squamous cell carcinoma, hand	66	M	11	------	------
5	Squamous cell carcinoma, lip and tonsil	66	M	------	8½	------
6	Squamous cell carcinoma, tongue	61	M	------	------	7½
7	Squamous cell carcinoma, larynx	59	F	------	------	11
8	Carcinoma, breast	58	F	------	------	10
9	"	63	F	------	------	14
10	"	62	F	------	------	10
11	"	52	F	8	------	------
12	"	59	F	------	------	9
13	"	54	F	------	------	9
14	"	69	F	------	------	12½
15	"	73	F	10½	------	------
16	"	61	F	------	------	9½
17	"	43	F	------	------	13½
18	"	70	F	------	------	12
19	"	76	F	------	------	9½
20	"	78	F	------	8½	------
21	"	44	F	------	------	9
22	"	68	F	------	------	9
23	Sarcoma, breast	78	F	------	------	16
24	Bronchiogenic carcinoma	45	M	------	------	11
25	"	45	M	------	------	7½
26	"	58	M	------	------	11½
27	"	51	M	------	------	11
28	"	53	F	------	------	10½
29	"	42	M	16½	------	------
30	"	52	M	11½	------	------
31	"	58	F	8	------	------
32	"	51	M	9½	------	------
33	"	58	M	------	12½	------
34	"	48	M	------	------	11

TABLE 6.—*Patients with malignant neoplastic diseases*—Continued

No.	Diagnosis	Age	Sex	Results in minutes according to solutions used		
				1, 2	3	4, 5, 6
35	Bronchiogenic carcinoma	49	M			10
36	"	55	M			9½
37	"	62	M		12½	
38	"	71	M			11
39	"	44	M		17	
40	Squamous cell carcinoma, esophagus	50	M			12
41	"	74	M		9½	
42	Carcinoma, stomach	76	M			13
43	"	57	M		9	
44	"	74	F	11		
45	"	79	M			12
46	"	43	F			13
47	"	73	F			10
48	Carcinoma, duodenum	69	M	12		
49	Carcinoma, cecum	41	M			13
50	Carcinoma, colon	72	M	14½		
51	"	67	M			22½
52	"	61	F			11
53	"	39	F			9
54	"	55	M	13½		
55	"	64	M	14		
56	"	83	M		13½	
57	"	67	M	8½		
58	Carcinoma, rectum	70	F			10½
59	"	83	M			11½
60	"	67	F			6½
61	"	65	F			10
62	Carcinoma, rectum and basal cell carcinoma, skin	73	M			14½
63	Carcinoma, rectum	74	M	10½		
64	"	62	M	8		
65	"	81	F			11½
66	"	81	M			15
67	"	57	M			7½
68	"	66	M			10
69	"	57	M			8½
70	"	67	M			11
71	"	76	M			9½
72	"	70	F			9½
73	"	70	M			10
74	"	72	M			13
75	"	50	M	10½		
76	"	58	F			10
77	"	61	M			5
78	"	79	M			11
79	"	59	M			10½
80	"	78	F			12½
81	"	60	M			12
82	Carcinoma, ampulla vater	36	F			9½
83	Carcinoma, pancreas	66	M	18		
84	"	70	M	12		
85	"	68	M			8½
86	"	61	M	11½		
87	"	61	M			9½
88	"	71	M		8	
89	Carcinoma, cervix	54	F			9½
90	"	69	F			10½
91	"	53	F			13
92	"	80	F			16
93	"	54	F	8½		
94	"	54	F		10	
95	"	44	F			13

TABLE 6.—*Patients with malignant neoplastic diseases*—Continued

No.	Diagnosis	Age	Sex	Results in minutes according to solutions used		
				1, 2	3	4, 5, 6
96	Carcinoma, uterus	47	F			13
97	"	59	F			10½
98	"	71	F			17
99	"	53	F			9
100	Carcinoma, kidney	66	M			14
101	Carcinoma, bladder	47	M			8½
102	"	35	M	14½		
103	"	71	M			13
104	"	74	M			14½
105	Carcinoma, prostate	74	M			13
106	"	77	M			12½
107	"	76	M			11
108	"	78	M	7½		
109	"	66	M			11½
110	"	80	M		11½	
111	"	81	M			4½
112	"	67	M			11½
113	"	74	M		8½	
114	"	69	M	16		
115	"	62	M			10½
116	"	59	M			10½
117	"	71	M	9		
118	"	63	M	9½		
119	"	70	M			7½
120	Mucoepidermoid carcinoma, parotid gland	22	F			10½
121	Carcinoma, submaxillary gland	80	M			8½
122	Carcinoma, thyroid gland	71	M	10½		
123	Metastatic carcinoma, type undetermined	59	M			13½
124	"	55	F			11
125	"	48	F			11
126	"	71	M	19		
127	"	48	F			10½
128	"	48	M	13½		
129	"	68	F			10
130	"	48	M			10½
131	"	41	F			12
132	"	66	M			9½
133	Melanoma, origin unknown	30	M	7		
134	Melanoma, cheek	70	F			12
135	Melanoma, foot	54	M	12½		
136	Fibrosarcoma, retroperitoneal	75	M	9½		
137	Hodgkin's disease	47	M	10½		
138	Lymphosarcoma	65	M			11½
139	"	57	M			11
140	"	69	M	6½		
141	"	82	F			13½
142	Myelogenous leukemia, chronic	33	M	13½		
143	Multiple myeloma	61	F	5½		
144	Ewing's sarcoma, sternum	25	F			8½

TABLE 7.—*Patients with benign neoplastic diseases*

No.	Diagnosis	Age	Sex	Results in minutes according to solutions used		
				1, 2	3	4, 5, 6
1	Lipoma, axilla	49	F			9
2	Lipoma, back	40	F			9½
3	Lipoma, axilla	65	M			9
4	Lipoma, thigh	42	F			11½
5	Cavernous lymphangioma, peritoneum	53	F		9	
6	Papilloma, larynx	36	M			12
7	Fibro-adenoma, breast	28	F		9½	
8	"	28	F			9
9	Cystadenoma, breast	28	F			9½
10	Intraductal papilloma, breast	21	F		8	
11	"	45	F			11
12	"	69	F			11
13	"	73	F			11
14	Meningioma	50	M		7	
15	"	62	F			9½
16	Giant follicular lymphoma	18	M	5½		
17	Enchondroma, phalanx	44	F			10
18	Osteoma, iliac bone	22	M			9
19	Adenoma, rectum	71	M			10½
20	Adenoma, thyroid gland	26	F			10
21	"	44	F			10½
22	Cystadenoma, ovary, and leiomyoma, uterus	59	F			8
23	Leiomyoma, uterus	47	F			9
24	"	32	F			8½
25	"	40	F			14
26	"	42	F			9½
27	"	39	F			9½
28	"	54	F			10
29	"	46	F			12
30	"	55	F			11
31	"	40	F			10½
32	"	36	F			9½
33	"	53	F			13
34	"	39	F			9½
35	"	65	F			14½

Table 8.—Patients with miscellaneous diseases

No.	Diagnosis	Age	Sex	Results in minutes according to solutions used		
				1, 2	3	4, 5, 6
1	Generalized syphilis	22	M	9		
2	"	27	F	8½		
3	"	33	F	7½		
4	"	45	M	9		
5	"	50	M	10¼		
6	"	56	M	8½		
7	"	58	F	11½		
8	Breast abscess	21	F			11
9	Cystic disease of breast	28	F			10½
10	"	43	F		8	
11	"	46	F			11½
12	"	47	F			11½
13	Cystic disease of breast and endometrial hyperplasia	53	F			10

Table 8.—Patients with miscellaneous diseases—Continued

No.	Diagnosis	Age	Sex	Results in minutes according to solutions used		
				1, 2	3	4, 5, 6
14	Cystic disease of breast	44	F	------	------	12½
15	"	48	F	------	------	11½
16	"	38	F	------	------	7
17	"	21	F	------	------	13
18	"	39	F	------	------	10½
19	Intraductal hyperplasia of breast	42	F	------	------	10
20	Osteitis deformans	83	M	------	9½	------
21	Fracture, zygoma	42	M	7½	------	------
22	Fracture, humerus	23	M	9	------	------
23	Fracture, femur	30	M	10	------	------
24	"	31	M	9	------	------
25	"	25	M	10½	------	------
26	"	51	M	9	------	------
27	Fracture, tibia and fibula	22	M	8½	------	------
28	Fracture, tibia and ulna	36	M	8	------	------
29	Fracture, tibia and fibula	43	M	10	------	------
30	Fracture, metatarsals	37	M	8½	------	------
31	Dislocation, cervical spine	36	M	9	------	------
32	Fracture, vertebral column	64	M	------	------	7½
33	Osteomyelitis, femur	41	M	8½	------	------
34	Diaphragmatic hernia	72	F	------	15½	------
35	Bronchiectasis	21	F	------	------	10½
36	"	46	F	12½	------	------
37	Chronic bronchitis	65	M	------	7	------
38	Asthma	52	F	10½	------	------
39	Pneumonia	59	M	15	------	------
40	Bronchopneumonia	77	M	------	------	10
41	Pneumonitis	43	M	------	------	8
42	Pulmonary tuberculosis	28	M	8	------	------
43	"	29	M	7	------	------
44	"	29	M	7	------	------
45	"	47	M	9	------	------
46	"	53	M	8	------	------
47	"	55	M	7	------	------
48	"	55	M	8	------	------
49	"	55	M	7	------	------
50	"	60	M	10½	------	------
51	"	62	M	7½	------	------
52	"	68	M	7	------	------
53	"	70	M	8	------	------
54	"	44	M	10	------	------
55	Hypertensive cardiovascular disease	46	M	------	------	8½
56	"	54	M	9½	------	------
57	"	65	M	10½	------	------
58	"	70	M	10	------	------
59	"	71	M	7½	------	------
60	Hypertensive cardiovascular disease and duodenal ulcer	58	M	7	------	------
61	Hypertensive cardiovascular disease and morphine addiction	42	M	8½	------	------
62	Hypertensive cardiovascular disease	47	M	------	------	8½
63	"	54	F	------	------	6
64	Thrombo-angiitis obliterans	70	M	8	------	------
65	Arteriosclerotic heart disease with angina	41	F	4½	------	------
66	"	77	M	------	------	8½
67	Arteriosclerotic heart disease	69	M	------	8	------
68	"	64	M	7½	------	------
69	"	74	M	------	9	------
70	"	77	F	13½	------	------
71	Coronary thrombosis	57	F	------	------	12½
72	Pernicious anemia	81	F	------	------	9½
73	"	67	F	13	------	------

Table 8.—Patients with miscellaneous diseases—Continued

No.	Diagnosis	Age	Sex	Results in minutes according to solutions used		
				1, 2	3	4, 5, 6
74	Hyperplasia, lymph node	11	M			8
75	Cervical lymphadenitis	28	M			9½
76	Acute suppurative lymphadenitis	41	F			9½
77	Chronic tonsillitis	20	M	9		
78	"	33	F			9
79	Gastric ulcer	64	M			13½
80	"	68	M			10½
81	"	52	M	7		
82	Chronic gastritis	68	M			10½
83	Duodenal ulcer	51	M		8½	
84	Perforated duodenal ulcer	58	F			9½
85	Duodenal ulcer	58	M			10
86	"	62	M			9
87	"	63	M			8
88	Duodenal diverticulum	78	F			9½
89	Peri-duodenal adhesions	62	M			9½
90	Amebic colitis	57	F			12
91	Infectious hepatitis	23	M	6½		
92	"	67	M			9
93	Laennec's cirrhosis	39	M	11		
94	"	46	M	11		
95	"	67	M	10½		
96	Liver abscess	38	M			9½
97	"	56	M			11½
98	Acute cholecystitis	55	F			11½
99	"	79	M		13	
100	Chronic cholecystitis	69	F		9	
101	"	74	F	9		
102	"	75	F			10
103	Steatorrhea	62	M			10
104	Internal hemorrhoids	26	M	8		
105	"	31	F			10
106	Chronic glomerulonephritis	49	M	21		
107	Chronic suppurative pyelonephritis	73	M	9½		
108	Pyelonephritis	25	M			11½
109	Acute pyelonephritis	61	M			11½
110	Pyonephrosis	48	F			10½
111	Nephroureterolithiasis	39	M			11½
112	Calculus, ureter	56	M			9
113	Calculus, bladder	70	M			11
114	Cystitis	62	M			9
115	Stricture, urethra, and calculus, bladder	66	M			11
116	Cystocele and rectocele	53	F			12½
117	Rectovaginal fistula	40	F			9
118	Benign hypertrophy of prostate	67	M			10½
119	"	67	M			10
120	"	68	M			10½
121	"	71	M	8½		
122	"	85	M			16
123	"	74	M		9	
124	"	60	M			12½
125	"	70	M			10
126	Tubo-ovarian abscess	30	F			12
127	"	16	F			11
128	Chronic salpingitis	25	F			11
129	Atrophy, endometrium	72	F			10½
130	Chronic endometritis	48	F		6½	
131	"	36	F			11
132	Chronic cervicitis	19	F		7½	
133	"	51	F			8½
134	"	37	F			10
135	"	18	F			9

Table 8.—Patients with miscellaneous diseases—Continued

No.	Diagnosis	Age	Sex	Results in minutes according to solutions used		
				1, 2	3	4, 5, 6
136	Prolapse, uterus, and chronic cervicitis	42	F	------	------	9½
137	Hyperplasia, endometrium	34	F	------	------	11
138	"	30	F	------	------	9½
139	"	40	F	------	------	11½
140	"	42	F	------	------	9½
141	Adenomyosis, uterus	39	F	9	------	------
142	"	40	F	------	------	12
143	"	44	F	------	------	10½
144	"	48	F	12	------	------
145	Retention of secundines	36	F	------	------	9
146	Hyperthyroidism	60	F	------	------	10½
147	Diabetes mellitus	55	F	------	------	8½
148	"	62	M	7½	------	------
149	"	74	M	8½	------	------
150	"	36	M	7½	------	------
151	"	27	M	5½	------	------
152	"	38	M	6½		------
153	"	56	M	6	------	------
154	"	75	M	4½	------	------
155	Multiple sclerosis	19	M	8	------	------
156	"	29	M	8½	------	------
157	Meningeal adhesions due to trauma	38	M	------	7½	------
158	Subdural hematoma	27	M	------	8	------
159	Grand mal epilepsy and chronic alcoholism	46	M	11	------	------
160	Cerebral hemorrhage	54	M	11½	------	------
161	"	65	M	11	------	------
162	Cerebral infarction	59	M	------	------	10
163	Myositis ossificans	17	M	------	------	10½
164	Retroperitoneal tuberculosis	47	M	------	------	10½
165	Synovial cyst, foot	33	M	------	------	9
166	Dacrocystitis	59	F	------	------	9
167	Severe cold	43	M	------	------	7½

STUDIES OF VARIOUS TESTS FOR MALIGNANT NEOPLASTIC DISEASES. II. THE GRUSKIN INTRADERMAL TEST [1] [2]

LOUISE WIEGENSTEIN, M. D., and RAYMOND F. HAIN, M. D., *Department of Pathology, University of Washington School of Medicine, Seattle, Wash.*

In 1932, Gruskin (*1*) reported an intradermal test for the diagnosis of carcinoma, using as an antigen an extract of epithelial cells from the liver, pancreas, or submaxillary glands of fetal calves or sheep of less than 2 months' gestation. Gruskin felt that a foreign protein of embryonic character is present in malignant cells and is also found in the circulating blood and fixed tissue cells. Intradermal injection of an antigen prepared from embryonic tissue, he stated, should result in an allergic reaction in a patient with carcinoma. His criterion for a positive test was the appearance within 15 minutes of a slight area of inflammation at the site of injection of the antigen, with pseudopod formation at the margin. Physiological saline was first injected as a control. Gruskin performed the test (with 100 percent accuracy) on 29 patients on whom a definite diagnosis of cancer had already been made or was subsequently confirmed.

MATERIALS AND METHODS

In repeating this work, the original directions for the preparation of the antigen and the testing technique were followed in every detail. Three separate antigens were used: "A" was prepared from pooled embryo calf liver, "B" from pooled embryo sheep liver, and "C" from pooled embryo sheep pancreas and submaxillary gland. The procedure was as follows:

The embryos were obtained at a local abattoir. The criteria for age (as recommended by inspectors from the United States Bureau of Animal Husbandry) were a length of 6 inches, or less, and lack of hair. The liver antigens were then prepared in the following manner, using sterile equipment and technique throughout:

The abdominal cavities were opened and approximately 5 gm. of liver from several embryos were dissected out and pooled in a Petri dish. As much of the capsule as possible was removed; the tissue was washed with sterile distilled water, cut into small pieces, and transferred to a 250 cc.

[1] Received for publication September 14, 1950.

[2] This investigation was aided by grants from the National Cancer Institute, The American Cancer Society, and the Damon Runyon Fund.

bottle. Fifty cubic centimeters of sterile distilled water was added, and the mixture shaken vigorously for 30 minutes. Any aggregates of fibrous tissue were then removed as completely as possible by the use of fine forceps, and the mixture was placed in 50-cc. centrifuge tubes and centrifuged at 3,000 r. p. m. for 5 minutes. The supernatant fluid was decanted, and the sediment was washed twice with approximately 20 cc. of sterile distilled water. After the second washing the mixture was again centrifuged at 1,000 r. p. m. for 3 minutes and the supernatant fluid decanted. The remaining sediment was transferred to a mortar and rubbed up with 20 times its volume of sterile N/10 NaOH, added 1 cc. at a time; the alkaline extract was poured into 2 50-cc. centrifuge tubes and allowed to stand at room temperature for 24 hours. The tubes were then centrifuged for 5 minutes at 1,000 r. p. m., and a measured amount of supernatant fluid was pipetted into an Erlenmeyer flask. The pH was adjusted to 7.0 with N/10 HCl, using nitrazine paper as an indicator. When a pH of 7.0 was reached, 2 tryptose-broth tubes and a tube of sterile physiological saline (as a control for turbidity) were inoculated; the remaining solution was placed in rubber-stoppered vaccine vials and stored in the refrigerator. The cultures were examined at the end of 24 and 48 hours and showed no growth.

To prepare antigen "C" approximately 1 gm. of pancreas and submaxillary gland was removed from several sheep embryos, using sterile technique; it was then placed in a Petri dish, covered with hypotonic saline solution, and put into a freezing chamber until partially frozen. The fibrous tissue and ducts were then teased away and the remaining epithelial tissue macerated with approximately 5 cc. of physiological saline, then transferred to a 50-cc. centrifuge tube and centrifuged at 2,500 r. p. m. for 5 minutes. The supernatant fluid was poured off, and the sediment was placed in a 37° C. oven until the cells were rather doughy. Any remaining fluid was then poured off and the cells shaken up with ether. The ether was decanted, and the cells were allowed to stand at room temperature for 5 minutes. The remaining sediment was rubbed up with 25 times its volume of N/10 NaOH and allowed to stand 24 hours at room temperature. The mixture was then centrifuged at 1,000 r. p. m. for 10 minutes; a measured amount of supernatant fluid was pipetted into a flask and neutralized to a pH level of 7.0 with N/10 HCl, cultured, and bottled in the same manner as the liver antigens.

The patients selected for testing were those who had definite evidence of carcinoma—either clinically outspoken, inoperable or incompletely resectable at the time of surgery, postoperative or postradiation recurrence, or demonstrable metastases. The diagnosis in each case was substantiated by biopsy, surgery, or autopsy. In performing the test 0.2 cc. of physiological saline was injected intradermally with a #27 hypodermic needle into the skin of the inner aspect of the forearm, taking 15 to 20 seconds for injection. About 3 inches below the site of the saline injection, 0.2 cc. of antigen was then injected with the same technique. At the end of a 15-minute period the test was read independently by

2 observers. The distribution of the neoplasms in the patients tested is shown in table 1.

TABLE 1.—*Distribution of neoplasms*

Primary site of neoplasm	Number of cases	Primary site of neoplasm	Number of cases
Bronchus	2	Prostate	11
Larynx	1	Testicle	1
Stomach	9	Breast	7
Colon	4	Skin	3
Rectum	6	Tongue	2
Pancreas	1	Mouth	3
Ovary	2	Tonsil	2
Cervix	1	Pharynx	1
Kidney	2		
Bladder	2	Total	60

A total of 60 patients, 23 of whom had known metastases, was tested, with the following results: 11 showed reactions which according to Gruskin's description were interpreted as being positive; *i. e.*, an area of inflammation with marginal pseudopods at the site of injection of the antigen; 22 showed merely an irregular area of erythema, varying from 1.0 to 3.5 cm. in diameter; 17 showed a regular wheal, with slight to no erythema, varying from 0.6 to 1.2 cm. in diameter; and 10 showed no reaction.

In each case, the clinical status of the patient with regard to degree of emaciation, dehydration, jaundice, edema, and temperature elevation, as well as the condition of the skin at the site of injection, was evaluated at the time the test was performed. In all 10 patients failing to react, a 1–2 plus dehydration or emaciation (on a 1–4 plus basis) was noted. No correlation was possible with the remaining patients. In every case the 2 independent readings were in agreement.

Since the majority of the patients complained of marked stinging or burning during the injection of the antigen, the question arose as to how much of the swelling and erythema might be due to chemical irritation from the solutions used in preparing the antigenic extract rather than to a true allergic response. Accordingly, a second control was prepared, consisting of N/10 NaOH neutralized to a pH level of 7.0 with N/10 HCl, using nitrazine paper as an indicator, which corresponded to the procedure for extraction and neutralization of the antigen. It was felt that this solution, which was approximately 0.1 N, represented a better control than saline, with a normality of 0.15. At a later date 8 of the 11 patients who had given a positive test were retested, using both saline and neutralized controls and all 3 antigens. The results are shown in table 2. Four of the patients showed no reaction with saline, but showed an irregular erythema without pseudopod formation with the neutralized control and each of the 3 antigens. Two showed irregular erythema without pseudopod formation at the site of all 5 injections. The remaining 2 patients showed no reaction at the site of injection of either control, but erythema and definite pseudopod formation with 3 antigens.

It was felt that these 2 patients probably represented true positives. It is of further interest that 1 of these 2 patients gave a negative reaction to 2 antigens when the Hoff-Schwartz (*2*) intradermal test was performed some time later. In the first 6 patients it was concluded that the reaction was due to chemical irritation and did not represent a true allergic response.

TABLE 2.—*Results of retesting, using saline and neutralized controls*

Primary site of neoplasm	Saline Control	Neutralized Control	Antigen A	Antigen B	Antigen C	Result	
						Observer 1	Observer 2
Bronchus	0	±	±	±	±	Neg.	Neg.
Breast	±	±	±	±	±	Neg.	Neg.
Breast	0	±	±	±	±	Neg.	Neg.
Breast	0	0	+	+	+	+	+
Stomach	±	±	±	±	±	Neg.	Neg.
Stomach*	0	0	+	+	+	+	+
Colon	0	±	±	±	±	Neg.	Neg.
Prostate	0	±	±	±	±	Neg.	Neg.

*=Hoff-Schwartz negative
0=No reaction
±=Irregular erythema; no pseudopods
+=Irregular erythema; definite marginal pseudopods

SUMMARY AND CONCLUSIONS

1. The Gruskin intradermal test for carcinoma was re-evaluated and the original directions were followed as closely as possible.

2. A greater number and wider variety of patients were tested in this series than in the original series.

3. Of 60 patients with definitely proved carcinoma, only 2 gave definitely positive tests.

4. These results are obviously at variance with those of the original author and are sufficiently poor to preclude any possible usefulness of the tests as performed in this experiment for purposes of cancer diagnosis.

REFERENCES

(*1*) GRUSKIN, B.: Intradermal test for determination of malignancy. J. Lab. & Clin. Med. 17: 1237–1243 (1932).

(*2*) HOFF, F., and SCHWARTZ, K.: Über Haut und Krebs und über intrakutane Krebsdiagnostik. München. med. Wchnschr. 71: 816–818 (1924).

STUDIES OF VARIOUS TESTS FOR MALIGNANT NEOPLASTIC DISEASES. III. THE HOFF-SCHWARTZ INTRADERMAL TEST [1] [2]

LOUISE WIEGENSTEIN, M. D., and RAYMOND F. HAIN, M. D., *Department of Pathology, University of Washington School of Medicine, Seattle, Wash.*

The intracutaneous injection of serum obtained from cancer patients who had received irradiation therapy has been reported by Hoff and Schwartz (*1*) as producing a diagnostic reaction in patients with cancer. Using 7 different sera taken at varying intervals from 3 patients—1 with carcinoma of the bladder, 1 with carcinoma of the cervix, and 1 with melanoma—26 tests were performed on 14 patients, each with a known carcinoma. Of these tests, 23 were positive and 3 negative. The 3 negative results occurred in 2 moribund patients who had previously shown a positive reaction. Of 24 tests on 17 noncancer patients, 19 were reported negative and 5 positive. Of the 5 positive reactions 1 occurred in a patient with an aleukemic lymphadenosis, 1 in a patient with pernicious anemia and 3 in a patient on whom no definite diagnosis had been established, although she was thought to have had "old adhesions of the uterine adnexa due to peritonitis." The specific amount of radiation therapy to the donor in each case was not mentioned. The authors merely state, "the patients were subjected in the usual way to therapeutic irradiation; the sera were diagnostically useful when they were taken between 3 hours and 3½ months after irradiation: those sera used the longest were useful 4 months after they were obtained; normal serum was used as a control; the characteristic reaction was the appearance of a violet spot at the injection site of the test serum; and the optimum time for reading the test is 48 hours."

MATERIALS AND METHODS

In repeating the work of Hoff and Schwartz their exact procedure was followed as closely as was feasible. Seven different sera from 5 patients were used for antigens. One patient had a carcinoma of the urinary bladder and 4 had carcinoma of the uterine cervix. It was impossible, as would now be expected, to find a patient with melanoma who was receiving radiation therapy. The amount of radiation that had been

[1] Received for publication September 14, 1950.

[2] This investigation was aided by grants from the National Cancer Institute, The American Cancer Society, and the Damon Runyon Fund.

received by the time serum was obtained and the interval from the onset of therapy to the date the blood was drawn are shown in table 1.

TABLE 1.—*Amount of radiation received and time interval involved*

Patient	Site of neoplasm	Antigen	Amount of radiation received by time serum was obtained	Interval from onset of therapy
				Days
A	Bladder	Bl I	2,750 r	13
B	Cervix	Cx I	7,000 r; 4,980 mg. hrs	33
C	"	Cx II	3,800 r; 770 mg. hrs	24
		Cx IIa	4,000 r; 1,542 mg. hrs	64
D	"	Cx III	7,800 r; 6,070 mg. hrs	44
		Cx IIIa	7,800 r; 6,070 mg. hrs	85
E	"	Cx IV	2,400 r	6

Under sterile conditions, 15–30 cc. of blood was withdrawn from each donor. The blood was centrifuged within 30 minutes at 1,500 r. p. m. for 1/2 hour and the serum placed in 5 cc. sterile vaccine bottles and stored in the refrigerator. In accordance with Hoff's directions the tests were carried out as follows: 0.1 cc. of normal serum was injected intracutaneously into the skin over the fascia lata of the thigh. A No. 27 hypodermic needle was used and sufficient force was exerted on the plunger of the syringe to produce a small, firm wheal. A similar injection of the test serum was made 5 cm. caudad to the control. Whenever possible, 2 test sera were used and injections were always made in duplicate, about 7 cm. apart, so that the majority of the patients had 6 injections. At the end of 48 hours the results of the tests were read independently by 2 observers.

A total of 98 tests was carried out on 60 patients. All patients showed evidence of carcinoma at the time of testing, and all diagnoses were confirmed by biopsy or subsequent surgery or autopsy. Only 1 patient gave a positive reaction, and to 2 different test sera (fig. 1). All other tests were entirely negative, according to the criteria described by Hoff and Schwartz. In no instance was there any difference of opinion by the 2 observers. Table 2 shows the distribution of the neoplasms in the cases studied.

TABLE 2.—*Distribution of neoplasms*

Primary site of neoplasm	Number of cases	Primary site of neoplasm	Number of cases
Larynx	1	Prostate	4
Bronchus	3	Penis	1
Stomach	4	Testicle	1
Colon	8	Breast	7
Rectum	5	Skin	1
Pancreas	3	Mouth	1
Gall Bladder	1	Palate	1
Ovary	2	Tongue	2
Cervix	7	Thyroid	1
Uterus	3		
Bladder	3	Total	60
Kidney	1		

Of interest is the fact that 1 other patient, clinically thought to have carcinoma of the cervix, gave a faintly positive reaction to 2 different test sera. This patient is not included in the series, however, as a later biopsy was negative for carcinoma. It is also of interest that the 1 patient who gave a positive reaction had shown a negative reaction with the Gruskin intradermal test for carcinoma (*2*).

A control series of normal individuals and of patients with other diseases was not tested, as the results obtained with the cancer patients were so uniformly negative.

SUMMARY AND CONCLUSIONS

1. The Hoff-Schwartz intradermal test for carcinoma was repeated on a series of 60 patients with definitely proved carcinoma, following the orginal method as closely as possible.

2. Of 98 tests carried out on these 60 patients, all were negative except for 1 patient who showed a positive reaction to 2 different antigens.

3. These results are obviously at variance with those of the original author and are sufficiently poor to preclude any possible usefulness of the tests as performed in this experiment for purposes of cancer diagnosis.

REFERENCES

(*1*) Hoff, F., and Schwartz, K.: Über Haut und Krebs und über intrakutane Krebsdiagnostik. München. med. Wchnschr. 71: 816–818 (1924).

(*2*) Gruskin, B.: Intradermal test for determination of malignancy. J. Lab. & Clin. Med. 17: 1237–1243 (1932).

PLATE 77

FIGURE 1.—Positive tests in patient with adenocarcinoma of the pancreas. The upper circle marks the site of injection of the control serum; the lower two, of the test sera.

Wiegenstein and Hain

929548—51——9

STUDIES OF VARIOUS TESTS FOR MALIGNANT NEOPLASTIC DISEASES. IV. THE EFFECT OF ZINC ION UPON THE SERUM ALKALINE PHOSPHATASE ACTIVITY [1]

LESTER D. ELLERBROOK, STUART W. LIPPINCOTT, and H. DAVIS CHIPPS, *Clinical Trials Unit, Cancer Control Branch, National Cancer Institute, Bethesda, Md., and Department of Pathology, School of Medicine, University of Washington, Seattle, Wash.*

In 1946 Roche *et al.* (*1*) reported that a minute concentration of zinc ion usually had an inhibitory effect upon the serum alkaline phosphatase activity of cancer patients while it usually activated this enzyme in the sera of other individuals. Because of the favorable results reported by these authors, and because their approach differed from that utilized in many so-called cancer tests, it was decided to investigate this phenomenon under carefully controlled conditions. The purpose of the study was to determine whether the measurement of these effects would be of value as either a diagnostic or a screening test for the detection of malignant neoplastic disease.

Roche *et al.* (*1*) had used a modification of the Bodansky method to determine the enzyme activity in the sera of 16 normal individuals, 85 noncancer patients, and 82 cancer patients, both in the absence and presence of 10^{-5} M zinc sulfate. The sera of 96 percent of the noncancer subjects showed enzyme activation in the presence of zinc ion (mean approximately 25 percent; range 0–80 percent). The enzyme was inhibited by zinc ion in 85 percent of the 182 sera obtained from the 82 cancer patients (mean inhibition approximately 15 percent; range 55 percent inhibition to 25 percent activation).

In 1947 Roche and his associates (*2*) stated "the addition of zinc ion in minimal concentration ($ZnSO_4$ M/10,000) weakly activates alkaline phosphomonoesterase of serum in normal and in many pathological human subjects, but not in cancer patients. On the contrary, in the latter cases a slight inhibition of the enzymatic activity is almost always observed following the addition of zinc ion to the serum." They also stated that the serum phosphatase of cancer patients regains the property of being activated by zinc ion following removal of the neoplasm. The effects of different concentrations of zinc ion were demonstrated by the use of

[1] Received for publication September 26, 1950.

929548—51——10

M/10,000, M/5,000, M/1,000 and M/500 concentrations in the study of sera of 43 noncancer subjects, 109 cancer patients studied before operation, and 25 cancer patients tested at least 10 days after surgery. With the M/10,000 concentration the sera of 80 percent of the noncancer group showed activation and none showed inhibition. The sera of 9 percent of the cancer patients showed activation and 67 percent showed inhibition. The sera of 94 percent of the postoperative cancer patients showed activation, while none showed inhibition. In general there was more inhibition in each group as the concentration of zinc ion was increased. Just why the cancer sera showed less inhibition with M/10,000 zinc ion than had previously been found with one-tenth as much zinc is not clear. It was mentioned that although Kay's method for the determination of phosphatase activity had been used in some of their earlier work, the method of Bodansky was used in this study because incubation of the enzyme-substrate mixture for 3 hours at pH 9.2 was preferable to incubation for 16 hours at pH 7.4 [actually the Bodansky procedure commonly employed (*3*) involves incubation for 1 hour at pH 8.6]. These authors concluded that the "possibility of applying our results to the diagnosis of cancer could be determined only in conjunction with histologic studies of the tumors whose existence appears to control to a varying extent the inhibition of phosphatase by very low concentrations of zinc."

During the course of our study 2 reports of similar studies were published. O. Bodansky and Blumenfeld (*4*) used a modification of the method of A. Bodansky (*3*) to study the effect of 10^{-5}, 10^{-4} and 10^{-3} M $ZnSO_4$ upon the serum alkaline phosphatase activity in groups of 14 to 25 individuals without cancer (normal and patients without malignancy) and in groups of 22 to 39 patients with cancer. They found no significant difference between the mean values for the enzyme inhibitions of the individuals without cancer and the patients with cancer at any of the 3 concentrations of zinc sulfate. There was no inhibition with 10^{-5} M, about 10 percent with 10^{-4} M and about 50 percent with 10^{-3} M zinc sulfate.

Fishman, Wayne, and Homburger (*5*) used Bodansky's 1932 procedure (*6*) to study the effect of zinc ion upon the serum phosphatase activity of a total of 23 normal individuals and of 22 patients with advanced cancer. They reported, "the behavior of the cancer and noncancer groups was found to be similar both qualitatively and quantitatively." They concluded by stating, "serum alkaline phosphatase from subjects with and without cancer was inhibited by zinc sulfate (1×10^{-5} M) to the same extent." Just how they arrived at these conclusions from the study of such small groups of individuals is not clear. A study of the individual results presented in their tables 1 and 2 indicates either that there are typographical errors, or that the control sera and the cancer sera were not studied with the same concentrations of zinc sulfate. Eight normal sera were studied by incubation for 1 hour with 1×10^{-5} M zinc ion, 3 were incubated 1 hour with 4×10^{-6} M Zn^{++}, 12 were incubated for 3 hours with 1×10^{-5} M Zn^{++} and 7 of these were also incubated for 3

hours with 4×10^{-6} M Zn^{++}. Six cancer sera were incubated for 1 hour with 1×10^{-6} M Zn^{++}; 2 of these were also incubated for 1 hour with 4×10^{-5} M Zn^{++}. Six other cancer sera were incubated for 3 hours with 1×10^{-6} M Zn^{++} and 3 of these, plus 5 other sera, were incubated for 3 hours at 4×10^{-5} M zinc ion. It is interesting to note that Roche *et al.* (*1*) found a mean activation of about 30 percent with normal sera tested with 1×10^{-5} M zinc sulfate, Bodansky and Blumenfeld found no effect (normal plus noncancer patients) and Fishman *et al.* found a mean inhibition of 51 percent with 8 sera incubated for 1 hour and 64 percent with 12 sera incubated for 3 hours.

In our work the Shinowara, Jones, and Reinhart (*7*) modification of the Bodansky (*3*) method was used for the estimation of phosphatase activity, since this method involves the incubation of enzyme (serum) and substrate at approximately pH 9.3, the optimum for serum alkaline phosphatase activity. In this method the serum inorganic phosphorus concentration is determined both before and after incubation with a buffered sodium beta glycerophosphate substrate. The activity of the enzyme is estimated from the amount of phosphorus liberated during incubation for 1 hour. The increase in inorganic phosphorus concentration (in mg. per 100 ml. of serum) is expressed as units of phosphatase activity. The sensitivity and specificity of the zinc ion effect were studied by determining the alkaline phosphatase activity of the fresh fasting serum of normal individuals, of patients with benign and malignant neoplastic diseases, and of patients with other miscellaneous diseases by incubation of the serum and substrate in the absence of added zinc ion and in the presence of zinc ion at concentrations of 10^{-5} M and 10^{-4} M. In addition, studies were made of the reproducibility of the results obtained, of the possibility of using fasting and postprandial specimens interchangeably, and of the effect of refrigeration of the sera before testing. The effect of the pH of incubation was also studied by making some determinations at the lower pH of about 8.6–8.8 used by Bodansky and at a still lower pH of approximately 8.3.

SELECTION OF SUBJECTS FOR TESTING

Previously established criteria and methods for the selection of subjects and collection of blood specimens, set forth in the first paper of this series (*8*), were adhered to in the present work: all patients with neoplastic diseases were tested prior to treatment, and the diagnoses were confirmed by microscopic examination of tissues obtained at biopsy, operation, or autopsy. None of these patients had received a blood transfusion within 1 week prior to testing.

One hundred and thirty-one normal individuals, ranging in age from 18 to 66 years, were used as a control group. The patients with neoplastic diseases included 29 with benign and 92 with malignant lesions. Careful review of the clinical and laboratory records of each individual with malignant disease indicated that 51 had metastases. One hundred and thirty patients with miscellaneous nonneoplastic diseases were tested for

comparison with the other groups. Every individual tested had fasted overnight before the blood specimen was taken.

REPRODUCIBILITY

The precision of the method used is probably not as great as might be expected for several reasons. A micro technique was used throughout (0.200-ml. specimen of serum for each part of the test) in order to conserve serum for other tests. The determination of the phosphatase activity consists of two parts (the estimation of serum inorganic phosphorus both before and after incubation with substrate) each of which is subject to error. The effect of each concentration of zinc was measured by still another test, which again was subject to error. An estimate of the usual reproducibility of the tests is afforded by a series of 57 tests of 22 cancer sera performed by a total of 5 technicians. The inorganic phosphorus concentrations of the sera ranged from 2.7 to 11.3 mg. per 100 ml. and the phosphatase activities ranged from 2.3 to 16.9 units per 100 ml. The coefficients of variation for the 2 determinations were 5.6 percent and 6.7 percent, respectively.

FASTING *VERSUS* POSTPRANDIAL SPECIMENS

The possibility of using postprandial blood specimens instead of fasting specimens was studied by simultaneously testing the fasting serum and that obtained 2 hours after breakfast from 2 normal individuals and 22 cancer patients. The average postprandial serum inorganic phosphorus concentration was 5.2 percent (0.225 mg. per 100 ml.) less than the corresponding fasting value. This difference was significant at the 5-percent level. The average postprandial phosphatase activity was 3.1 percent (0.229 unit per 100 ml.) greater than the corresponding fasting value, although this difference was not significant at the 5-percent level. The enzyme activity of the postprandial specimens was inhibited 5.9 percent in the presence of 10^{-5} M zinc ion, while the inhibition was 2.0 percent for fasting sera. This difference in inhibitions was significant at the 5-percent level. In the presence of 10^{-4} M zinc ion the average inhibition with postprandial sera was 35.7 percent, while that found with fasting sera was 25.1 percent. This difference was barely significant at the 5-percent level. Because of these observed differences all routine tests were made upon fasting serum.

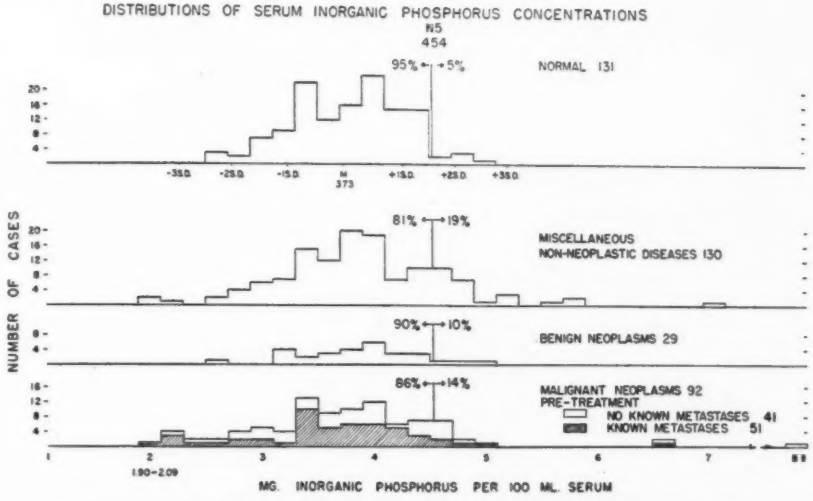
REFRIGERATION

The possibility of testing sera after storage in the refrigerator was studied by testing 1 normal serum and 27 specimens obtained from cancer patients both on the day the specimen was obtained and again after refrigeration for 24 hours. The latter values differed from the original average values as follows: the inorganic phosphorus concentration was 3.2 percent greater (0.143 mg. per 100 ml.). This difference was significant at the 1-percent level. The phosphatase activity was 1.9 percent greater, the inhibition by 10^{-5} M zinc ion was 2.5 percent less, and the inhibition

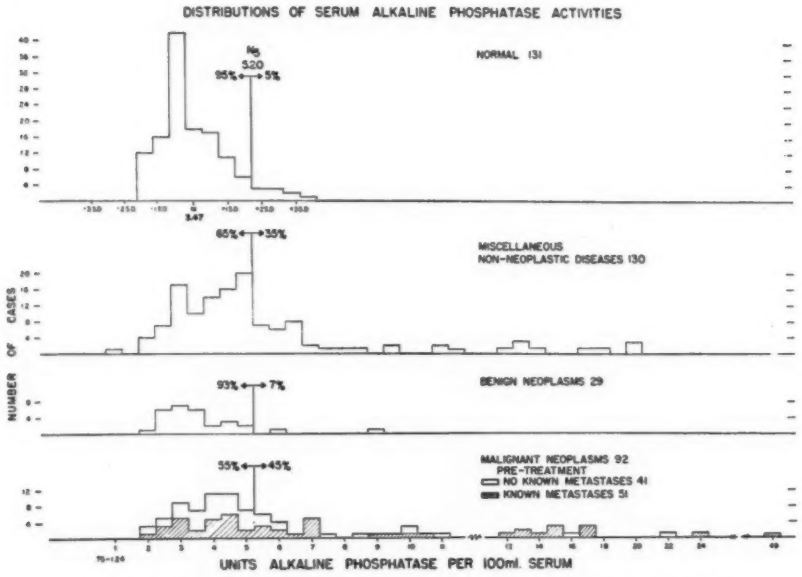
by 10^{-4} M zinc ion was 2.9 percent greater. None of these last 3 differences were significant at the 5-percent level. In spite of this fact it was decided to include in this report only the results obtained by use of fresh specimens obtained from individuals in the fasting state.

SENSITIVITY AND SPECIFICITY OF TEST

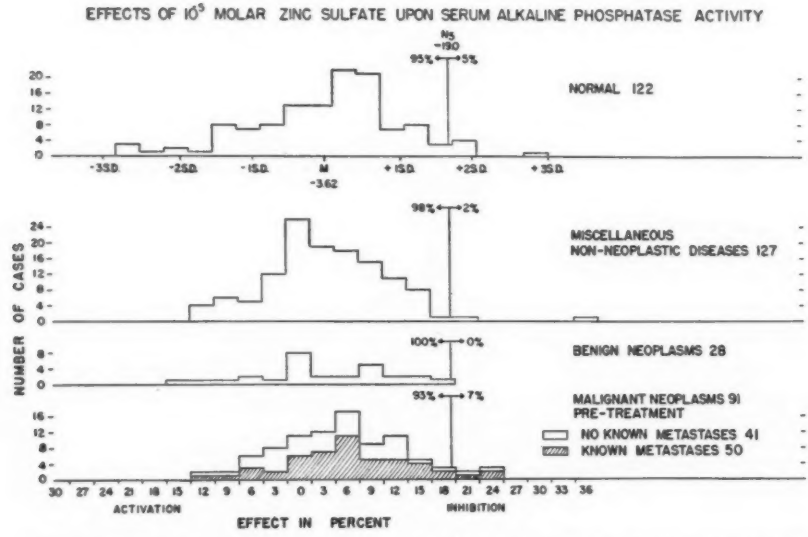
The results obtained for the serum inorganic phosphorus concentration, the alkaline phosphatase activity and the percentage activation or inhibition by 10^{-5} M and 10^{-4} M zinc sulfate are shown in text-figures 1–4. It can be seen that the serum inorganic phosphorus concentrations of the three groups of patients did not differ markedly from normal. The alkaline phosphatase activity, however, tended to be slightly increased in some of the patients with miscellaneous nonneoplastic diseases, and to a somewhat greater degree in a considerable number of the individuals with malignant neoplasms. Thirty-five percent of the individuals with miscellaneous diseases had serum alkaline phosphatase values exceeding 95 percent of the normal values (this 95 percent point, N_5, is calculated as the mean value plus 1.645 standard deviations). Further study of these values made by grouping the miscellaneous diseases into several types of pathologic processes (table 1) showed abnormalities of from 25 to 50 percent except in the group with hyperplasia, in which 16 percent were abnormal. These percentages were not found to differ significantly among themselves, assuming the true percentage in each group to be the over-all percentage of 35. In these particular groups abnormal values do not appear to be of diagnostic aid. Forty-five percent of the cancer patients had serum alkaline phosphatase values exceeding 95 percent of the normal values. Further study of these results was made by classifying



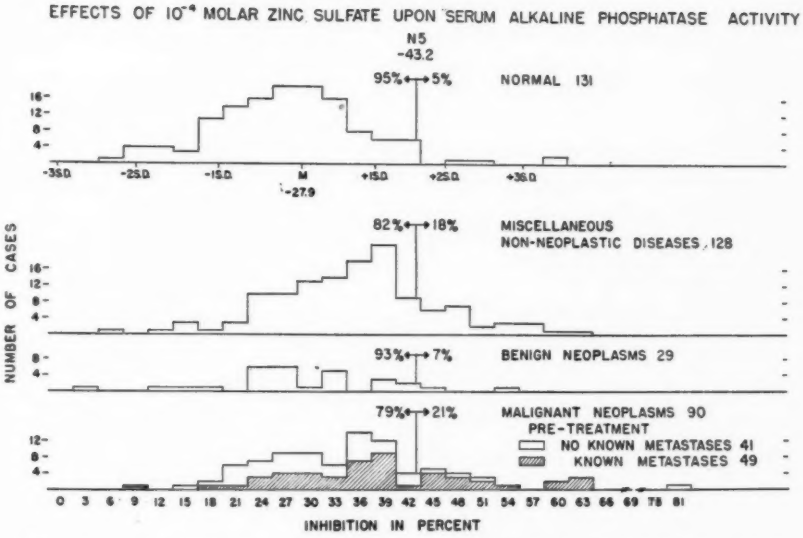
TEXT-FIGURE 1.—Distributions of serum inorganic phosphorus concentrations.



TEXT-FIGURE 2.—Distributions of serum alkaline phosphatase activities.



TEXT-FIGURE 3.—Effects of 10^{-5} molar zinc sulfate upon serum alkaline phosphatase activity.



TEXT-FIGURE 4.—Effects of 10^{-4} molar zinc sulfate upon serum alkaline phosphatase activity.

TABLE 1.—*Summary of results obtained in miscellaneous diseases*

	Serum alkaline phosphatase								
	Units			Inhibition by zinc					
				M/100,000			M/10,000		
	Number cases	Number abnormal	Percent abnormal	Number cases	Number abnormal	Percent abnormal	Number cases	Number abnormal	Percent abnormal
Lesions that might clinically be confused with cancer:									
Hyperplasia	19	3	16	18	0	0	19	1	5
Gastrointestinal lesions	10	5	50	10	0	0	10	0	0
Inflammation (chronic)	19	6	32	19	0	0	19	4	21
Other pathologic processes:									
Tuberculosis and syphilis	15	4	27	14	0	0	15	4	27
Circulatory diseases	15	7	47	15	0	0	14	1	7
Metabolic disorders	8	2	25	8	0	0	8	3	38
Trauma	12	6	50	12	0	0	11	2	18
Others	32	13	41	31	2	6	32	8	25
Total	130	46	35	127	2	2	128	23	18

the patients into histogenetic types of malignant neoplastic diseases, and according to presence or absence of metastases (table 2). In the groups with carcinomas of undetermined origin and with bronchiogenic carcinomas 83 and 78 percent of the results were abnormal while, in contrast, in the group with breast carcinomas only 8 percent were abnormal. The percentages of abnormal results found in the six groups differ significantly (at the 1-percent level) from the over-all percentage of 45, and therefore each group is governed by its own rate. It should be noted, however, that because of the small number of cases in each group the observed percentages are subject to large sampling errors. The cases with metastases showed a definitely higher percentage of abnormal values than those without known metastases, the results being 55 and 32 percent, respectively. In the group with benign neoplasms 7-percent abnormal values were noted. These results indicate that the phosphatase values may be elevated in a number of miscellaneous diseases, and in some types of malignant neoplastic diseases; but these abnormalities are of no apparent diagnostic value in the latter group.

TABLE 2—*Summary of results obtained in neoplastic diseases prior to treatment*

	Serum alkaline phosphatase								
	Units			Inhibition by zinc					
				M/100,000			M/10,000		
	Number cases	Number abnormal	Percent abnormal	Number cases	Number abnormal	Percent abnormal	Number cases	Number abnormal	Percent abnormal
Malignant:									
Gastrointestinal	28	11	39	27	2	7	28	9	32
Breast	12	1	8	12	1	8	12	1	8
Bronchiogenic	9	7	78	9	1	11	9	2	22
Prostate	8	5	63	8	1	13	8	0	0
Carcinoma, type undetermined	6	5	83	6	0	0	6	0	0
All other types	29	12	41	29	1	3	27	7	26
Total	92	41	45	91	6	7	90	19	21
Known metastases	51	28	55	50	3	6	49	15	31
No known metastases	41	13	32	41	3	7	41	4	10
Benign	29	2	7	28	0	0	29	2	7

The effect of 10^{-5} M zinc sulfate during the incubation period was far different from that reported by Roche *et al.* (*1*). Instead of finding an activation of normal serum phosphatase the mean inhibition in the 122 normal sera studied was 3.62 percent, and 95 percent of the inhibitions were less than 19 percent (5 percent were 19 percent or over). The mean inhibition of the 91 cancer sera was 5.50 percent, and 7 percent were 19 percent or over. The mean inhibition of the 28 benign-neoplasm sera was

3.11 percent, and no inhibitions were 19 percent or over. The mean inhibition of the 127 miscellaneous-disease sera was 3.68 percent, and 2 percent were 19 percent or over. With this concentration of zinc sulfate, there were no significant differences detected between phosphatase activity in normal serum and that in malignancy serum, based upon comparisons of mean values, variation between individuals and the percentages detected as positive. In the group of miscellaneous diseases separated into processes and in the group of malignant neoplasms divided on a basis of histogenesis, no notable increase in inhibition was found in any category (tables 1 and 2).

The effect of 10^{-4} M zinc sulfate during the incubation period was definitely inhibitory to enzyme activity in all groups studied. The mean inhibition in the 131 normal sera studied was 27.9 percent, and 95 percent of the inhibitions were less than 44 percent (5 percent were 44 percent or over). The mean inhibition of the 90 cancer sera was 35.7 percent, and 21 percent were 44 percent or over. The mean inhibition of the 29 benign-neoplasm sera was 29.5 percent, and 7 percent were 44 percent or over. The mean inhibition of the 128 miscellaneous disease sera was 35.6 percent, and 18 percent were 44 percent or over. In general, with this concentration of zinc sulfate there was a significant difference between normal serum and malignancy serum. The greater variance of the cancer values and the greater mean inhibition were both significant at the 5-percent level. The difference between the 21 percent of abnormal values in the cancer group and the 5 percent in the normal group was significant at the 1-percent level. Table 1 shows that in the miscellaneous diseases the greatest number of abnormal results was 38 percent, found in the small group with metabolic disorders, while 1 group with gastrointestinal lesions showed no abnormal results. In the group with hyperplasia only 5 percent abnormal results were found. Table 2 shows that abnormal results in groups of malignant diseases ranged from no abnormal values in carcinomas of the breast, of the prostate, and of the undetermined type, to 32 percent abnormalities in gastrointestinal carcinomas. However, for both the miscellaneous and the malignant diseases, the abnormal percentages in the various groups did not differ significantly from their over-all percentages, 18 and 21, respectively. The group with metastases showed a significantly higher percentage of abnormal values than those without known metastases, the results being 31 and 10 percent, respectively. Individual test results and diagnoses for all patients are listed in tables 5, 6, and 7 (see Appendix).

DISCUSSION

Just why our results differ from those of Roche is not known. It is possible that our group of cases of malignancy differs from his groups of patients, but that still does not explain why 10^{-5} M zinc activated the enzyme in less than half of our normal sera. It was thought that perhaps

the reason was the difference between his particular modification of the Bodansky method and the Shinowara, Jones, and Reinhart modification used by us. Roche *et al.* (*2*) stated that they used Bodansky's method (no reference) which involves incubation of serum and substrate for 3 hours at pH 9.2. Bodansky published several modifications of his original test but the procedure most widely used is probably that published in the Journal of Biological Chemistry, 101: 93–104 (1933). In that method the buffered substrate was a mixture of sodium beta glycerophosphate and sodium barbital. The pH was said to be 8.6± 0.15 after the serum (itself a buffer) was added. In the recent paper of Bodansky and Blumenfeld (*4*) it is stated that in the 1933 procedure the usual pH of the mixture of serum and unadjusted buffered substrate is about 8.75 ± 0.05. They noted that the addition of zinc sulfate to give the desired concentration caused a decrease in pH, and consequently they adjusted the pH to 8.75 in each determination in which zinc sulfate was present. Several textbooks in describing Bodansky's method advise the adjustment of the pH of the buffered substrate to 8.6 before addition of the serum—the final pH presumably would be lower.

The exact pH of incubation is of importance in the measurement of serum alkaline phosphatase activity, and definitely higher values are obtained at the optimum pH of approximately 9.2 used by us. It was considered possible, however, that the use of a lower pH might enable us to duplicate Roche's results, although he specifically stated that he incubated at pH 9.2. For this reason a number of sera were examined both in the presence and absence of zinc under our usual conditions (incubation pH 9.2—9.3), under the conditions outlined by Bodansky in 1933 (unadjusted buffer, final pH 8.6 ± 0.15), and under the conditions listed in several textbooks in which the buffered substrate is first adjusted to pH 8.6, and the final pH of incubation at 37° C. is approximately 8.3.

The effects of zinc ion and of serum upon the pH of the three substrates are shown in table 3. Also demonstrated are the effects of temperature and the effect of zinc ion when added to the serum-buffered substrate

TABLE 3.—*Effect of serum and of zinc sulfate upon the pH of buffered substrates*

	pH at room temperature		
Substrates	10. 55	9. 60	8. 65
Substrates + 10^{-5} M $ZnSO_4$	9. 25	9. 02	8. 58
Substrates + 10^{-4} M $ZnSO_4$	8. 70	8. 98	8. 57
Substrates + serum (cancer and normal)	[1] 9. 50	[2] 9. 10	[1] 8. 60
	pH at 37° C.		
Substrates + serum (cancer and normal)	9. 20	8. 80	8. 30
Substrates + serum + 10^{-5} M $ZnSO_4$	8. 85	8. 60	8. 23
Substrates + serum + 10^{-4} M $ZnSO_4$	8. 84	8. 58	8. 16

[1] 11 and 8 sera.
[2] 2 and 4 sera.

mixtures. It can be seen that the addition of the smaller amount of zinc decreased the pH values of the substrates themselves (least effect at pH 8.6) and that additional zinc sulfate caused a further appreciable pH drop only with the pH 10.6 substrate. The same tendencies were noted upon the addition of zinc sulfate to serum-substrate mixtures although the pH changes were smaller. The 37° C. pH values of the two zinc-serum-substrate mixtures were nearly identical in each case. Consequently it appears that the decrease in phosphatase activities upon increasing the concentrations of zinc from 10^{-5} M to 10^{-4} M is not due to differences in pH.

Since Bodansky and Blumenfeld had found no significant differences between the sera of cancer patients and those of other individuals when tested at pH 8.75, we performed simultaneous tests upon normal, cancer, and miscellaneous-disease sera by incubation at our usual pH of 9.2 and at pH 8.2. Twenty-three normal sera were studied, the mean values and standard deviations were determined and the 95th percentile points were calculated. The percentages of results beyond these points (greater than 95 percent of the normal values) obtained in tests of sera from 48 cancer patients and from 20 patients with miscellaneous diseases are shown in table 4.

TABLE 4.—*Comparison of average results obtained by incubation at pH 9.2 and at 8.2*

	Normal		Cancer		Miscellaneous	
	pH 9.2	pH 8.2	pH 9.2	pH 8.2	pH 9.2	pH 8.2
Number of sera	23	23	48	47	20	20
Alkaline phosphatase units per 100 ml.	4. 3	1. 8	8. 0	3. 5	5. 3	2. 1
Percent "abnormal":						
10^{-5} M zinc ion	5. 0	5. 0	$\frac{5}{48}=10\%$	$\frac{5}{47}=11\%$	$\frac{1}{20}=5\%$	0
10^{-4} M zinc ion	5. 0	5. 0	$\frac{8}{48}=17\%$	$\frac{5}{47}=11\%$	$\frac{2}{20}=10\%$	0

It is apparent that although the enzyme activities (and the percentages of inhibition) were decreased at the lower pH value, the percentages of abnormal results found at pH 8.2 did not differ markedly from those found at pH 9.2.

SUMMARY AND CONCLUSIONS

1. The effect of 10^{-5} M and 10^{-4} M zinc sulfate upon the serum alkaline phosphatase activity as measured by the method of Shinowara, Jones, and Reinhart was studied in fresh sera of 382 fasting individuals. The groups included 131 normal individuals, 130 patients with various miscellaneous diseases, 29 patients with benign neoplasms, and 92 patients with malignant neoplasms of which 51 had metastasized. The 2 groups with neoplasms were tested before treatment. Serum alkaline phosphatase

values exceeding 95 percent of normal values were found in 35, 7, and 45 percent of these groups of patients.

2. The presence of 10^{-5} M zinc sulfate lowered the average pH of the incubation mixtures by 0.35 unit but the mean enzyme activities in the 4 groups of individuals were decreased by only 2–6 percent. This is in contrast to Roche's reported inhibition in cancer sera and activation in other sera.

3. The presence of 10^{-4} M zinc sulfate caused no further change in the pH values but the mean inhibitions in the 4 groups were 28, 36, 29, and 36 percent, respectively. Twenty-one percent of the cancer inhibitions and 18 percent of the miscellaneous-disease inhibitions were greater than 95 percent of the normal findings. This is in contrast to Roche's findings.

4. The percentages of abnormal inhibitions in cancer sera were rather similar in tests involving incubation at pH 9.2 and at pH 8.2.

5. The change of enzyme activity in the presence of 10^{-5} M and 10^{-4} M zinc sulfate is not sufficiently sensitive or specific for diagnostic or screening purposes in the detection of cancer.

REFERENCES

(*1*) Roche, J., Nguyen-van Thoai, Marcelet, J., and Desruisseaux, G.: L'action de l'ion zinc sur la phosphatase alcaline du sérum humain à l'état normal et dans divers états pathologiques en particulier chez les cancéreau. Compt. rend. Soc. de biol. 140: 632–634 (1946).

(*2*) Roche, J., Cornil, L., Desruisseaux, G., Baudoin, N., and Long, S.: Action de l'ion zinc sur la phosphatase alcaline du sérum humain et cancers. Compt. rend. Soc. de biol. 141: 1251–1252 (1947).

(*3*) Bodansky, A.: Phosphatase studies. II. Determination of serum phosphatase. Factors influencing the accuracy of the determination. J. Biol. Chem. 101: 93–104 (1933).

(*4*) Bodansky, O., and Blumenfeld, O.: Effect of zinc on serum alkaline phosphatase activity in patients with and without cancer. Proc. Soc. Exper. Biol. & Med. 70: 546–547 (1949).

(*5*) Fishman, W. H., Wayne, A., and Homburger, F.: The evaluation of diagnostic tests for cancer. II. Inhibition of serum alkaline phosphatase by zinc ion. Cancer Research 9: 681–683 (1949).

(*6*) Bodansky, A.: Phosphatase studies. I. Determination of inorganic phosphate—Beer's law and interfering substances in the Kuttner-Lichtenstein method. J. Biol. Chem. 99: 197–206 (1932).

(*7*) Shinowara, G. Y., Jones, L. M., and Reinhart, H. L.: Estimation of serum inorganic phosphate and "acid" and "alkaline" phosphatase activity. J. Biol. Chem. 142: 921–933 (1942).

(*8*) Eriksen, N., Ellerbrook, L. D., and Lippincott, S. W.: Studies of various tests for malignant neoplastic diseases. I. The reduction of methylene blue by plasma. J. Nat. Cancer Inst. 11:705–728 (1951).

APPENDIX

TABLE 5.—*Patients with miscellaneous diseases*

No.	Diagnosis	Age	Sex	Serum alkaline phosphatase			Serum inorganic phosphorus mg. per 100 ml.
				Units per 100 ml.	Percent activation (+) or inhibition (−) by zinc		
					M 100,000	M 10,000	
1	Generalized syphilis	45	M	3.8	8−	42−	4.7
2	"	50	M	5.0	2+	42−	3.4
3	"	56	M	5.0	8−	38−	3.8
4	"	58	F	7.4	3+	46−	3.6
5	Cystic disease of breast	28	F	2.8	3−	25−	3.7
6	"	43	F	3.3	12−	36−	3.8
7	"	46	F	3.4	0	35−	3.4
8	"	47	F	3.8	1−	48−	3.7
9	"	53	F	3.5	3−	26−	3.5
10	"	44	F	2.0	13−	32−	3.3
11	"	48	F	2.9	9+	35−	2.9
12	"	38	F	4.1	5−	24−	2.2
13	Intraductal hyperplasia of breast	42	F	2.9	15−	19−	3.2
14	Osteitis deformans	83	M	11.2	0	40−	3.7
15	Fracture, humerus	23	M	5.6	5+	36−	4.0
16	Fracture, femur	30	M	5.8	5−	36−	4.6
17	"	31	M	6.5	0	46−	3.5
18	"	25	M	4.5	9+	20−	3.8
19	"	51	M	6.0	5−	40−	3.9
20	Fracture, tibia and fibula	22	M	4.5	2+	47−	4.7
21	"	43	M	6.5	5−	40−	4.0
22	Fracture, tibia and ulna	36	M	6.4	2+	41−	3.3
23	Fracture, metatarsals	37	M	2.6	8+	38−	4.4
24	Dislocation, cervical spine	36	M	4.4	0	------	4.3
25	Diaphragmatic hernia	72	F	4.0	3+	48−	1.9
26	Bronchiectasis	21	F	3.6	5−	39−	3.9
27	"	46	F	3.3	0	60−	4.5
28	Chronic bronchitis	65	M	5.0	0	32−	3.3
29	Asthma	52	F	5.1	10−	49−	4.0
30	Bronchopneumonia	77	M	6.4	0	31−	4.3
31	Pneumonitis	43	M	4.5	18−	33−	3.6
32	Severe cold	43	M	3.2	22−	28−	3.6
33	Pulmonary tuberculosis	28	M	4.8	8−	25−	3.4
34	"	29	M	5.2	15−	40−	4.1
35	"	29	M	4.8	6−	35−	3.1
36	"	53	M	4.7	0	32−	4.1
37	"	55	M	5.7	3−	42−	3.0
38	"	55	M	3.2	3−	53−	4.8
39	"	55	M	3.5	11−	37−	3.7
40	"	60	M	4.0	12+	45−	4.0
41	"	62	M	3.4	3−	56−	3.7
42	"	68	M	4.9	------	36−	3.6
43	Hypertensive cardiovascular disease	46	M	5.1	4−	36−	3.5
44	"	65	M	5.2	6−	31−	3.1
45	"	70	M	5.8	5−	40−	2.8
46	"	71	M	5.0	2−	30−	3.5
47	Hypertensive cardiovascular disease and duodenal ulcer	58	M	14.4	8−	53−	4.2
48	Hypertensive cardiovascular disease and morphine addiction	42	M	6.8	6−	40−	4.6
49	Hypertensive cardiovascular disease	47	M	6.0	2−	22−	3.1
50	"	54	F	5.4	8−	13−	3.7
51	Arteriosclerotic heart disease with angina	41	F	3.8	11−	42−	4.4
52	"	77	M	2.5	12−	40−	2.8
53	Arteriosclerotic heart disease	69	M	4.4	0	39−	3.9
54	"	64	M	5.7	0	35−	3.0
55	"	74	M	1.1	0	------	3.5

TABLE 5.—*Patients with miscellaneous diseases*—Continued

No.	Diagnosis	Age	Sex	Serum alkaline phosphatase			Serum inorganic phosphorus mg. per 100 ml.
				Units per 100 ml.	Percent activation (+) or inhibition (−) by zinc		
					M 100,000	M 10,000	
56	Coronary thrombosis	57	F	3.3	7−	31−	4.9
57	Internal hemorrhoids	31	F	3.2	2−	27−	4.0
58	Pernicious anemia	81	F	2.9	36−	36−	3.8
59	"	67	F	4.3	11−	63−	4.7
60	Hyperplasia, lymph node	11	M	12.8	5−	37−	4.3
61	Acute suppurative lymphadenitis	41	F	5.1	------	37−	4.6
62	Chronic tonsillitis	33	F	6.0	9−	34−	4.4
63	Gastric ulcer	64	M	5.8	4−	41−	3.8
64	Chronic gastritis	68	M	7.2	3+	26−	3.8
65	Duodenal ulcer	51	M	4.1	7−	31−	3.8
66	Perforated duodenal ulcer	58	F	4.6	12−	38−	4.0
67	Duodenal ulcer	58	M	4.8	4−	33−	4.6
68	"	62	M	5.4	15−	39−	3.9
69	"	63	M	2.3	4−	7−	3.2
70	Diverticulum duodenum	78	F	3.4	6+	24−	4.0
71	Periduodenal adhesions	62	M	5.5	15−	37−	4.2
72	Amebic colitis	57	F	5.0	1−	38−	4.3
73	Infectious hepatitis	67	M	9.3	9−	48−	4.0
74	Laennec's cirrhosis	39	M	11.1	3−	56−	3.8
75	"	46	M	13.3	4+	39−	4.0
76	"	67	M	3.8	11+	40−	3.9
77	Acute cholecystitis	55	F	19.8	2−	43−	3.9
78	"	79	M	19.5	0	51−	3.0
79	Chronic cholecystitis	69	F	4.2	0	29−	3.3
80	"	74	F	20.5	1+	39−	3.2
81	"	75	F	16.9	8−	25−	4.7
82	Steatorrhea	62	M	3.2	6−	56−	3.3
83	Chronic glomerulonephritis	49	M	6.5	0	25−	4.4
84	Chronic suppurative pyelonephritis	73	M	9.3	11−	53−	5.8
85	Pyelonephritis	25	M	4.9	8+	47−	5.1
86	Acute pyelonephritis	61	M	4.7	2+	26−	2.6
87	Pyonephrosis	48	F	3.9	8−	33−	3.7
88	Calculus, kidney	39	M	4.9	6+	33−	5.2
89	Calculus, ureter	56	M	6.3	1−	30−	3.6
90	Calculus, bladder	70	M	18.3	0	39−	7.0
91	"	66	M	4.8	0	40−	4.6
92	Cystitis	62	M	5.2	7−	16−	3.4
93	Cystocele and rectocele	53	F	3.2	3−	15−	4.6
94	Rectovaginal fistula	40	F	2.4	8−	33−	3.7
95	Benign hypertrophy of prostate	67	M	2.2	6+	28−	3.1
96	"	67	M	3.2	10−	40−	2.8
97	"	68	M	3.8	16−	38−	5.7
98	"	85	M	5.2	3+	25−	3.4
99	"	74	M	2.6	8+	31−	1.9
100	"	60	M	4.0	------	30−	3.0
101	"	70	M	3.7	1−	28−	3.4
102	Tubo-ovarian abscess	30	F	4.5	1−	30−	4.7
103	"	16	F	6.4	9−	44−	4.3
104	Chronic salpingitis	25	F	3.1	11−	34−	4.7
105	Atrophy, endometrium	74	F	6.7	7−	48−	4.0
106	"	72	F	4.4	3−	38−	3.4
107	Chronic endometritis	48	F	4.3	14−	35−	4.4
108	Chronic cervicitis	51	F	4.4	4−	22−	4.5
109	"	37	F	2.3	13−	24−	3.6
110	Prolapse, uterus, and chronic cervicitis	42	F	2.2	14−	41−	3.3
111	Hyperplasia, endometrium	34	F	5.7	7−	30−	4.2
112	"	30	F	3.1	3+	23−	4.0
113	Adenomyosis, uterus	39	F	7.8	1+	36−	4.1
114	"	40	F	3.2	2+	31−	3.7

TABLE 5.—*Patients with miscellaneous diseases*—Continued

No.	Diagnosis	Age	Sex	Serum alkaline phosphatase			Serum inorganic phosphorus mg. per 100 ml.
				Units per 100 ml.	Percent activation (+) or inhibition (−) by zinc		
					M 100,000	M 10,000	
115	Adenomyosis, uterus	44	F	2.8	1−	26−	3.8
116	"	48	F	13.2	2+	42−	2.6
117	Retention of secundines	36	F	2.2	5+	36−	3.3
118	Hyperthyroidism	60	F	4.7	5−	28−	3.7
119	Diabetes mellitus	55	F	4.3	11−	33−	5.2
120	"	74	M	4.0	0	50−	4.6
121	"	36	M	5.2	4−	37−	4.0
122	"	27	M	3.8	8+	34−	4.0
123	"	38	M	3.1	13+	45−	3.6
124	"	56	M	4.6	0	44−	3.7
125	"	75	M	12.3	8−	34−	2.7
126	Meningeal adhesions due to trauma	38	M	2.8	11+	14−	4.2
127	Subdural hematoma	27	M	3.0	10−	33−	2.9
128	Myositis ossificans	17	M	8.3	4−	23−	5.5
129	Retroperitoneal tuberculosis	47	M	11.7	16−	29−	4.7
130	Synovial cyst, foot	33	M	2.5	5−	27−	3.4

TABLE 6.—*Patients with benign neoplastic diseases*

No.	Diagnosis	Age	Sex	Serum alkaline phosphatase			Serum inorganic phosphorus mg. per 100 ml.
				Units per 100 ml.	Percent activation (+) or inhibition (−) by zinc		
					M 100,000	M 10,000	
1	Lipoma, axilla	49	F	3.7	8−	38−	3.6
2	"	65	M	3.3	12−	16−	3.9
3	Lipoma, back	40	F	3.4	7−	27−	3.2
4	Cavernous lymphangioma, peritoneum	53	F	6.0	8+	53−	4.1
5	Meningioma	50	M	2.5	0	32−	4.9
6	"	62	F	9.0	8−	25−	4.2
7	Giant follicular lymphoma	18	M	5.1	0	39−	3.8
8	Enchondroma, phalanx	44	F	2.8	1−	33−	4.5
9	Osteoma, iliac bone	22	M	3.1	14+	4−	4.3
10	Fibro-adenoma, breast	28	F	2.6	12+	27−	3.3
11	"	28	F	3.1	2+	28−	3.9
12	Cystadenoma, breast	28	F	3.6	3−	23−	3.9
13	Intraductal papilloma, breast	21	F	3.5	9−	34−	4.0
14	"	69	F	4.2	1+	28−	3.3
15	"	73	F	3.1	7−	31−	2.6
16	Papilloma, larynx	36	M	2.8	1−	24−	3.5
17	Adenoma, thyroid	26	F	4.4	0	25−	3.1
18	"	44	F	2.0	2−	27−	3.6
19	Leiomyoma, uterus	47	F	4.7	0	34−	4.4
20	"	32	F	2.3	14−	25−	4.0
21	"	59	F	3.6	9−	44−	3.9
22	"	40	F	4.9	10−	24−	3.8
23	"	39	F	2.3	5+	41−	3.8
24	"	54	F	4.7	------	18−	3.7
25	"	46	F	4.1	12−	28−	3.1
26	"	55	F	2.9	6+	38−	4.7
27	"	40	F	2.5	14−	34−	4.3
28	"	34	F	2.9	1−	13−	4.1
29	"	53	F	2.5	17−	41−	3.2

TABLE 7.—*Patients with malignant neoplastic diseases*

No.	Diagnosis	Age	Sex	Metastasis		Serum alkaline phosphatase			Serum inorganic phosphorus mg. per 100 ml.
				Known	No	Units per 100 ml.	Percent activation (+) or inhibition (—) by zinc.		
							M/100,000	M/10,000	
1	Basal cell carcinoma, rectum	64	M		x	2.0	2+	22—	3.1
2	Squamous cell carcinoma, face	77	F		x	6.2	1—	21—	4.1
3	Squamous cell carcinoma, hand	66	M		x	4.0	5—	35—	2.9
4	Squamous cell carcinoma, skin	61	F		x	3.9	8—	49—	4.5
5	Squamous cell carcinoma, skin	92	M		x	10.9	3+	36—	2.1
6	Squamous cell carcinoma, lip and tonsil	66	M		x	3.3	3—	27—	3.9
7	Squamous cell carcinoma, larynx	59	F		x	3.5	2+	28—	4.0
8	Squamous cell carcinoma, tongue	61	M		x	4.8	9—	29—	2.8
9	Bronchiogenic carcinoma	51	M	x		9.5	8—	24—	5.0
10	"	53	F	x		5.4	24—	24—	4.1
11	"	58	F	x		23.8	5—	61—	6.5
12	"	51	M	x		4.5	7—	38—	3.5
13	"	48	M	x		17.4	16—	38—	3.8
14	"	55	M		x	10.0	3—	36—	4.7
15	"	62	M	x		4.4	2—	50—	3.3
16	"	71	M	x		7.1	6—	31—	2.8
17	"	44	M	x		15.1	1—	41—	3.9
18	Carcinoma, breast	62	F		x	4.6	11—	28—	3.7
19	"	52	F	x		3.2	16—	38—	4.2
20	"	59	F	x		4.7	6—	34—	3.4
21	"	54	F	x		4.1	5—	36—	3.3
22	"	69	F		x	3.6	8+	34—	2.6
23	"	73	F	x		5.5	7+	47—	2.5
24	"	61	F	x		4.0	7—	25—	3.8
25	"	70	F		x	3.1	0	30—	3.6
26	"	78	F		x	4.3	19—	28—	3.8
27	"	49	F	x		2.8	11—	22—	3.8
28	"	44	F	x		2.3	18—	32—	3.3
29	"	68	F	x		3.2	11+	10—	3.9
30	Squamous cell carcinoma, esophagus	50	M		x	3.2	0	41—	3.2
31	"	74	M		x	2.4	13—	42—	2.3
32	Carcinoma, stomach	76	M	x		10.0		26—	3.9
33	"	57	M		x	2.2	5+	82—	2.8
34	"	74	F		x	5.6	5—	44—	3.7
35	"	79	M	x		7.1	18—	37—	3.8
36	Carcinoma, duodenum	69	M	x		1.9	16—	63—	2.1
37	Carcinoma, colon	61	F		x	4.1	5—	32—	3.4
38	"	39	F	x		2.7	1+	17—	4.0
39	"	49	M		x	5.0	14—	32—	4.5
40	"	83	M		x	5.9	12—	51—	4.3
41	"	67	M	x		6.9	23—	36—	4.3
42	"	72	M	x		2.4	4—	50—	3.4
43	Carcinoma, cecum	41	M	x		5.8	0	35—	3.0
44	Carcinoma, rectum, and basal-cell carcinoma, skin	73	M	x		10.3	3—	32—	3.7
45	Carcinoma, rectum	62	M	x		3.8	3—	60—	3.5
46	"	81	F		x	5.1	4+	39—	3.2
47	"	67	M		x	3.9	5+	23—	3.6
48	"	76	M	x		4.4	10—	46—	2.1
49	"	70	F		x	3.2	6+	19—	4.5
50	"	83	M	x		9.0	7—	64—	3.3
51	"	70	M	x		6.9	5—	29—	3.2
52	"	72	M		x	7.3	21—	22—	4.4

TABLE 7.—*Patients with malignant neoplastic diseases*—Continued

No.	Diagnosis	Age	Sex	Metastasis: Known	Metastasis: No	Serum alkaline phosphatase: Units per 100 ml.	Percent activation (+) or inhibition (−) by zinc: M/100,000	Percent activation (+) or inhibition (−) by zinc: M/10,000	Serum inorganic phosphorus mg. per 100 ml.
53	Carcinoma, rectum	50	M	x		17.5	7−	53−	3.4
54	"	58	F		x	4.5	11+	24−	4.4
55	"	79	M		x	2.9	7−	22−	3.7
56	"	59	M	x		3.5	10−	35−	3.3
57	"	65	F		x	3.3	4+	20−	3.9
58	Carcinoma, pancreas	70	M	x		5.2	13−	49−	4.0
59	"	61	M	x		49.0	12−	40−	4.6
60	"	71	M		x	21.8	3−	35−	3.3
61	Carcinoma, cervix	53	F		x	8.3	13−	27−	4.4
62	"	80	F		x	16.2	11−	42−	4.5
63	"	54	F		x	4.5	7−	29−	4.5
64	"	54	F	x		4.5	20−	40−	4.4
65	"	44	F	x		4.0	8+	47−	4.0
66	Carcinoma, uterus	59	F		x	4.0	7−	29−	3.5
67	"	71	F		x	5.4	0	40−	8.8
68	"	53	F		x	4.2	8−	14−	4.0
69	Carcinoma, bladder	45	M	x		7.1	1+	44−	2.0
70	"	35	M	x		3.2	12−	44−	2.1
71	"	71	M		x	3.6	8−	24−	3.5
72	Carcinoma, prostate	78	M		x	10.2	3−	31−	3.0
73	"	66	M		x	5.2	1−	36−	6.5
74	"	80	M		x	5.0	4−	36−	3.9
75	"	74	M	x		3.0	3+	27−	3.3
76	"	62	M		x	5.4	12−	38−	3.9
77	"	59	M	x		12.4	2−	26−	4.2
78	"	63	M	x		17.3	8−	38−	3.6
79	"	70	M		x	2.6	23−	24−	3.0
80	Mucoepidermoid carcinoma, parotid	22	F		x	4.7	2+	36−	3.3
81	Carcinoma, submaxillary gland	80	M	x		3.8	1−		2.4
82	Carcinoma, thyroid	71	M	x		6.1	0	39−	4.3
83	Metastatic carcinoma, type undetermined	48	F	x		14.9	6−	37−	2.7
84	"	48	F	x		5.7	6+	27−	3.7
85	"	43	M	x		13.1	5+	40−	4.7
86	"	48	M	x		13.9	12−	31−	4.5
87	"	68	F	x		3.5	3−	36−	3.5
88	"	48	M	x		14.6	5−	29−	3.5
89	Sarcoma, breast	78	F	x		12.7	16−	46−	4.1
90	Hodgkin's disease	73	F	x		4.8	2−	62−	3.0
91	Multiple myeloma	61	F	x		6.5	10−	39−	3.3
92	Fibrosarcoma, retroperitoneal	75	M	x		4.4	2+		4.2

STUDIES OF VARIOUS TESTS FOR MALIGNANT NEOPLASTIC DISEASES. V. THE HEAT "COAGULATION" OF PLASMA[1]

NILS ERIKSEN, LESTER D. ELLERBROOK, EDWARD C. MEEK, and STUART W. LIPPINCOTT, *Clinical Trials Unit, Cancer Control Branch, National Cancer Institute, Bethesda, Md., and Department of Pathology, School of Medicine, University of Washington, Seattle, Wash.*

INTRODUCTION

This is one of a series of papers (*1–4*) reporting the results obtained in studies of certain tests proposed by other authors as useful aids in the diagnosis of malignant neoplastic diseases. These studies are being carried out in order to determine the sensitivity and specificity of each test and to assess its practical usefulness in the detection of cancer. In addition, studies are being made of certain technical factors that may be of importance in the routine use of a given test and in the interpretation of the observed results.

An inexpensive test technically simple enough to meet the needs of a mass screening program was reported by Black, Kleiner, and Bolker (*5*) in 1948. They observed that plasma from cancer patients tended to undergo heat "coagulation" more readily than plasma from normal individuals. Fibrinogen appeared to be a necessary factor in the reaction, because serum failed to show any increase in turbidity under the experimental heating conditions. Sixty-two percent of a series of 136 cancer cases were found to have plasma-coagulation values exceeding all but approximately 2 percent of those of 434 normal individuals. Fourteen percent of 77 cases of nonneoplastic diseases had elevated coagulation values. Elevations were found most frequently in active tuberculosis, rheumatic heart disease, and in late pregnancy. Thirteen percent of 15 cases of benign neoplasms showed abnormal values.

In another series of 473 cases of malignant neoplasms, Black and Speer (*6*) found 66 percent abnormal plasma-coagulation values. In 558 cases of nonneoplastic diseases, not including tuberculosis, rheumatic fever. or cirrhosis, only 9 percent abnormal values were observed. In 116 cases of nonmalignant neoplasms only 8 percent abnormal values were observed. These are apparently the same series of cases reported elsewhere by Black (*7*).

[1] Received for publication September 26, 1950.

Recently Kleiner, with Stettner and others (*8*), reported finding abnormal coagulation values in 80 percent of 72 cases of malignant diseases. In a series of 239 cases of nonmalignant diseases, approximately 10 percent of the coagulation values were found to be abnormal. Abnormal values were observed most frequently in cases of pregnancy, pulmonary tuberculosis, acute rheumatic fever, and quantitative disturbances of the blood proteins. Those cases with known plasma-protein abnormalities were excluded from consideration because it was felt that the results of the test in these subjects should be corrected for the concentration of protein present. No apparent consideration was given to abnormalities in plasma-protein concentrations occurring in patients with cancer.

The methods used and the results obtained in our study of the plasma-coagulation test are presented in the sections that follow.

SELECTION OF SUBJECTS FOR TESTING

Previously established criteria and methods for the selection of subjects and collection of blood specimens, set forth in the first paper of this series (*1*), were adhered to in the present work: all patients with neoplastic diseases were tested prior to treatment, and the diagnoses were confirmed by microscopic examination of tissues obtained at biopsy, operation, or autopsy. None of these patients had received a blood transfusion within 1 week prior to testing.

One hundred twenty-five normal individuals, ranging in age from 18 to 66 years, were used as a control group. The patients with neoplastic diseases included 54 with benign and 137 with malignant lesions. Careful review of the clinical and laboratory records of each individual with malignant disease indicated that 60 had metastases. One hundred forty-four patients with miscellaneous nonneoplastic diseases were tested for comparison with the other groups. Every individual tested had fasted overnight before the blood specimen was taken.

TECHNIQUE OF TEST

The experimental procedure of Black *et al.* (*5*) consists of the following steps: a) Measure 1 ml. of plasma (taken from a patient in a fasting state) into a Klett colorimeter tube and dilute to the 5-ml. mark with distilled water; b) take a reading of the light transmission in a Klett photoelectric colorimeter provided with a green filter (No. 54); c) immerse the tube at least as far as the 10-ml. mark in a vigorously boiling water bath for just 10 seconds, cool in running tap water, dry, and take a second reading of the light transmission. The difference between the first and second colorimeter readings is taken as the measure of heat coagulation or turbidity. The same technique was used by Stettner *et al.* (*8*) except that they specified immediate cooling of the tube for 30 seconds under cold running water.

In this work Black's technique was followed in all essential details, with especial care having been taken to cool the heated tubes immediately in cold running tap water, since a delay in cooling was shown to give higher and more variable results. Turbidity measurements were made

with a Coleman Junior spectrophotometer instead of a Klett photoelectric colorimeter, but this change, as Black points out, should not alter the qualitative nature of the coagulation-value distributions, although in general a different scale of numerical results may be expected. The heating of the diluted plasma was done in ungraduated Klett colorimeter tubes. One ml. of plasma[2] (fresh, fasting specimen) was measured from a volumetric pipette into the tube. Four milliliters of distilled water was added from a volumetric pipette. The liquids were mixed thoroughly, but without vigorous agitation. A portion of the mixture was then poured into a 10 by 75 mm. cuvette, and the apparent optical density was measured in the Coleman Junior spectrophotometer at $\lambda=540$ mμ. The portion was returned to the Klett tube, which was then immersed almost to its full length in a boiling water bath for 10 seconds, the time interval being measured with a stop watch. The tube was cooled immediately in cold tap water and rotated at the same time in order to mix its contents. The cuvette used for the initial density measurement was rinsed with a small portion of the mixture, and was then filled with a second portion. The apparent optical density was measured as before. The difference between the initial and final readings was recorded as the coagulation, or turbidity, value.

REPRODUCIBILITY OF RESULTS

The experimental error attached to a single plasma-coagulation value was estimated from a series of duplicate and replicate determinations on plasma specimens having a wide range of values. The results are shown in table 1. It is seen that the coefficient of variation, defined as the ratio of the standard error of measurement of a group of values to the mean coagulation value, decreases as the mean coagulation value increases. The combined data yield an average coefficient of variation of 7.3 percent, which means that there is a probability of 0.68 that a single result will be within approximately 7 percent of the true coagulation value, or, a probability of 0.95 that a single result will be within approximately 15 percent of the true value. This average estimate of error would of course be most reliable for coagulation values in the vicinity of the over-all mean, 0.048, of the values used in these computations.

TABLE 1.—*Reproducibility of results*

Mean coagulation value ($\bar{x}$)	Range of values	Number of specimens	Number of determinations per specimen	Estimated variance ($x\ 10^4$)	Estimated standard deviation (s)	Coefficient of variation ($s/\bar{x}$) (percent)
0. 0225	0. 007–0. 030	12	2	4. 92	0. 00222	9. 9
0.0367	. 028– . 052	17	2	9. 47	. 00308	8. 4
0. 0459	. 040– . 050	1	9	9. 11	. 00302	6. 6
0. 0480	. 043– . 053	1	9	9. 75	. 00312	6. 5
0. 0606	. 051– . 071	8	2	14. 81	. 00385	6. 4
0. 0797	. 073– . 089	1	10	18. 46	. 00429	5. 4
0. 0911	. 072– . 116	7	2	24. 79	. 00498	5. 5
0. 1008	. 098– . 107	1	4	18. 25	. 00427	4. 2
Over-all: 0. 048	. 007– . 116	48		12. 28	. 0035	7. 3

[2] Twenty milligrams of potassium oxalate per 10 ml. of blood was used to prevent clotting.

FRESH *VERSUS* REFRIGERATED SPECIMENS

Black *et al.* (*5*) recommended that the determination of the plasma-coagulation value be carried out on the day the sample is drawn because of their observation that the value falls as the plasma stands in the refrigerator for several days. Later Black (*7*), and also Stettner and associates (*8*), implied that a refrigeration period not exceeding 24 hours did not significantly affect the coagulation value. In our experience the coagulation value has generally undergone an appreciable decrease within the first 24 hours of storage in the refrigerator.

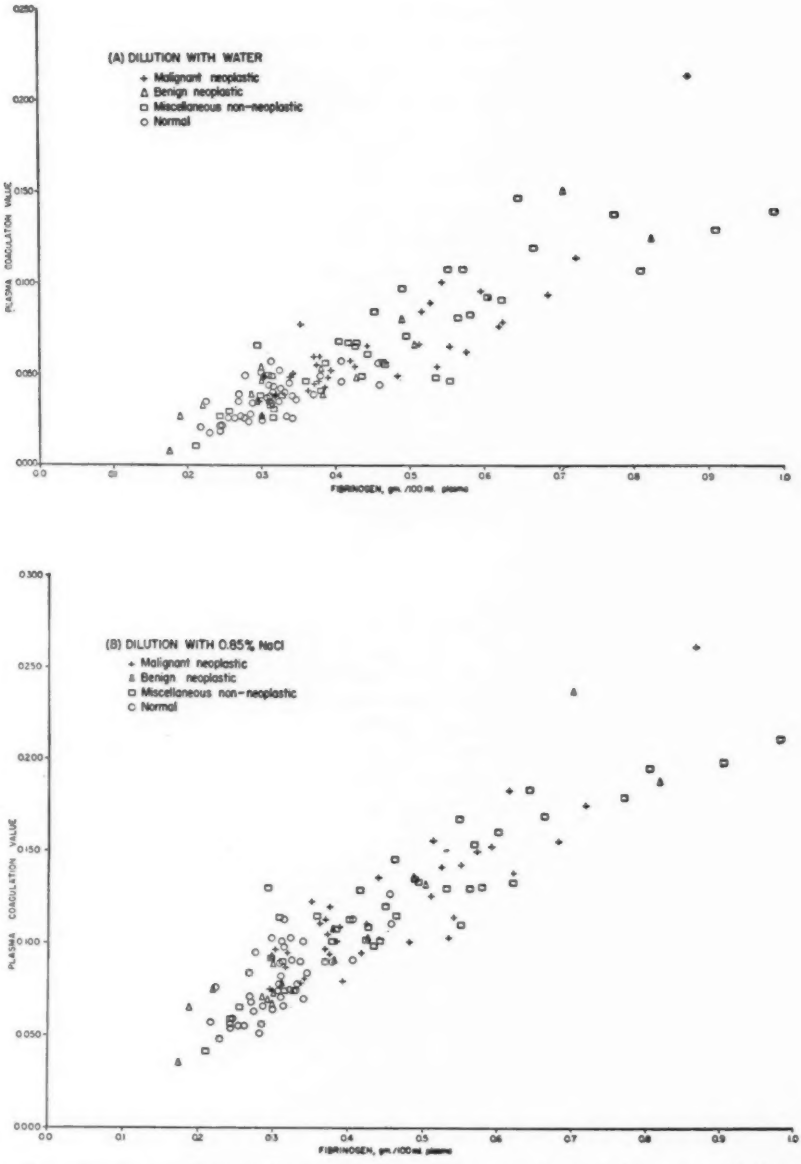
Coagulation values of certain plasma specimens were determined as soon as possible after the blood was drawn, and again after overnight storage of the specimens in the refrigerator. Such comparisons were made for plasmas taken from 100 cancer patients, 11 tuberculosis patients, and 9 normal individuals. Average decreases in the coagulation values of 21, 29, and 28 percent were observed in the respective groups. Each of these decreases is significant at the 1-percent level. For this reason all of our specimens were tested as soon as possible after they were drawn.

COAGULATION VALUE AS A FUNCTION OF FIBRINOGEN CONCENTRATION

Because of the obvious importance of fibrinogen in the coagulation reaction, a fibrinogen determination was done on practically every plasma specimen whose coagulation value was determined. Fibrinogen concentrations were determined by isolation of the fibrinogen as fibrin, according to the procedure of Cullen and Van Slyke (*9*), followed by direct nesslerization of a micro-Kjeldahl digest of the fibrin. Duplicate determinations on 12 plasma specimens with fibrinogen concentrations ranging from 0.205 to 0.517 gm. per 100 ml. gave a coefficient of variation of 3.9 percent.

Fibrinogen concentrations and the corresponding coagulation values for 132 plasma specimens are shown graphically in text-figure 1A. The points are scattered about a line that intercepts the fibrinogen axis between points representing fibrinogen concentrations of 0.1 and 0.2 mg. per 100 ml. Similar results were obtained by Black *et al.* (*5*). The 132 specimens represented in text-figure 1 are those that were tested by both the usual coagulation technique and a modified technique, in which the plasma was diluted with a 0.85 percent solution of sodium chloride instead of distilled water. Higher coagulation values were invariably obtained when saline was used as the diluent. The results are plotted against fibrinogen concentrations in text-figure 1B. The points are scattered about a line passing through or close to the origin.

Although in both cases the observed scatter appears to be greater than would be expected from experimental errors alone, the tendency toward a linear relationship between the fibrinogen concentration and the coagulation value is unmistakable. Aside from deviations resulting from experimental errors, quantitative changes in such factors as albumin, globulin, and inorganic salts could conceivably account for part of the



TEXT-FIGURE 1.—Relation between fibrinogen concentration and plasma-coagulation value.

A.—Dilution with water.
B.—Dilution with 0.85 percent sodium chloride.

observed deviations from a linear relationship. The pronounced effect of added saline on the coagulation value indicates that ionic strength may be one of these factors. Perhaps qualitative changes in the fibrinogen itself, affecting its heat-coagulation characteristics, are also responsible for some of the deviations.

SENSITIVITY AND SPECIFICITY OF THE TEST

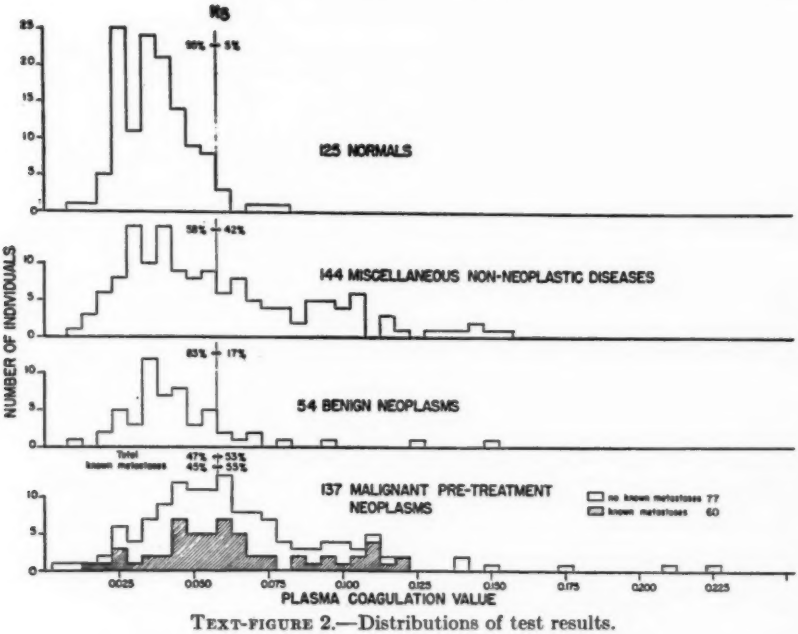
A critical coagulation value, N_s, differentiating between normal and abnormal test results, was calculated from the formula $N_5=m+1.645s$, where m is the mean of the group of normal values and s is the sample standard deviation. The statistical considerations underlying this choice of a critical value, which arbitrarily designates the highest 5 percent of the normal values as false-positive test results, have been presented by Dunn and Greenhouse (*10*).

The 125 normal individuals used for controls were from 18 to 66 years of age, with a median age of approximately 28. The mean coagulation value for the 18–28 age group (65 individuals; mean age, 24) was 0.0377; for the 29–66 age group (60 individuals; mean age 41) it was 0.0372. These means are obviously not significantly different. The variance among the coagulation values for the older age group was slightly greater than that for the younger group, but not significantly so at the 5-percent level. An analysis of the results for the age groups 18–29, 30–44, and 45–66, containing 72, 36, and 17 individuals, respectively, showed no significant differences among the mean coagulation values of the 3 groups. It is concluded that the factor of age would not appreciably bias results of the test applied to individuals of middle age. Because of the difficulty of obtaining a sufficient number of healthy, older individuals, no attempt was made to extend the analysis to include the later decades of life, although admittedly a large number of our cancer patients fell in this category.

The coagulation values for the 125 normal controls had a mean of 0.0375 and a standard deviation of 0.012, from which N_5 was computed to be 0.058. Any value equal to, or greater than, 0.058 was considered abnormal; *i. e.*, a positive test result.

The frequency of occurrence of abnormal values in the miscellaneous diseases and in the benign and malignant neoplasms is shown in text-figure 2. In addition, it was felt desirable to ascertain whether abnormal values might be particularly frequent for certain disease processes or, in the case of the malignant neoplasms, for particular sites of origin. Tables 2 and 3 therefore constitute a breakdown of the data for the patients contained in text-figure 2.

The results obtained in 191 cases of neoplastic diseases prior to treatment are shown in table 2. In the 54 cases of benign neoplasms studied, there were 17 percent positive tests, whereas in the group of 137 malignant neoplasms, there were 53 percent positive tests. In the various groups of malignant neoplasms there was considerable variation in the percentages of positive tests, with the smallest being 27 percent in the case of breast



TEXT-FIGURE 2.—Distributions of test results.

TABLE 2.—*Summary of results obtained in neoplastic diseases prior to treatment*

	Number of cases	Number abnormal	Percentage abnormal
Benign	54	9	17
Malignant:			
Gastrointestinal	40	22	55
Breast	15	4	27
Bronchiogenic	11	8	73
Prostate	12	9	75
Cervix and uterus	14	6	43
Type undetermined	9	7	78
All other types	36	16	44
Total	137	72	53
Known metastases	60	33	55
No known metastases	77	39	51

TABLE 3.—*Summary of results obtained in miscellaneous nonmalignant diseases*

	Number of cases	Number abnormal	Percentage abnormal
Hyperplasia	44	11	25
Gastrointestinal lesions	30	18	60
Inflammation (chiefly chronic)	29	10	34
Circulatory diseases	15	9	60
Others	26	12	46
Total	144	60	42

cancer and the largest 78 percent in those of undetermined type. Almost as large a percentage of positive results was found in the groups with bronchiogenic and prostatic cancer, namely, 73 and 75 percent respectively. However, no special significance should be attached to the relatively high proportions detected positive in these groups, except possibly for further exploration, since they are based on such small numbers. The proportions of positive results in all 7 categories were found not to differ significantly among themselves when the true rate is assumed to be the over-all rate of 0.53. The number of positive tests occurring in the groups with and without known metastases was essentially the same. Individual test results and confirmed diagnoses for all patients with neoplastic diseases are listed in tables 4 and 5 (see Appendix).

The results obtained in 144 cases of miscellaneous nonneoplastic diseases are shown in table 3. The over-all percentage of positive results was 42 percent, compared to 17 percent for the group with benign neoplasms and 53 percent for the group with malignant neoplasms. With reference to the several categories into which the miscellaneous diseases were separated, the lowest percentage of positive results, 25, was found in the hyperplasia group; the highest percentage, 60, was found in both the gastrointestinal and circulatory groups. The percentages of abnormal results found in these groups differ significantly (at the 5-percent level) from the over-all percentage of 42, and therefore each group has its own rate. It should be noted, however, that because of the small number of cases in each group the observed percentages are subject to large sampling errors. Individual test results and diagnoses for all patients with nonneoplastic diseases are listed in table 6 (see Appendix).

SUMMARY

The plasma coagulation test of Black has been applied to plasma specimens obtained from normal individuals and individuals having various neoplastic and nonneoplastic diseases. One hundred twenty-five normal individuals, ranging in age from 18 to 66 years, were tested. The average coagulation value of the older normals did not differ significantly from that of the younger normals. Coagulation values exceeding 95 percent of those predicted for a normal population were arbitrarily considered to be abnormal. Fifty-four individuals with benign neoplasms and 137 individuals with malignant neoplasms were tested before they had received any form of treatment, so far as it was possible to determine. Abnormal coagulation values were obtained in 17 percent of the group with benign neoplasms and in 53 percent of the group with malignant neoplasms. Sixty of the individuals with malignant neoplasms were known to have metastases; of these, 55 percent gave abnormal results. Of 144 individuals with miscellaneous nonneoplastic diseases, 42 percent gave abnormal results.

The standard error of a single measurement was found to be approximately 7 percent of the estimated true coagulation value.

A significant decrease in average coagulation values was observed in plasma specimens stored overnight in a refrigerator.

The magnitude of the plasma-coagulation value was found to be dependent upon the plasma-fibrinogen concentration.

CONCLUSION

The results obtained in this study are in agreement with the reports of Black and co-workers that the plasma-coagulation technique is not a specific diagnostic test for malignant neoplastic diseases. The results also show such low orders of over-all sensitivity and specificity in the detection of malignant neoplasms that the test would not be suitable for screening purposes.

Acknowledgment

The statistical analyses of data reported in this paper were carried out under the supervision of Mr. S. W. Greenhouse, Biometrics Section, National Cancer Institute.

REFERENCES

(*1*) Eriksen, N., Ellerbrook, L. D., and Lippincott, S. W.: Studies of various tests for malignant neoplastic diseases. I. The reduction of methylene blue by plasma. J. Nat. Cancer Inst. 11:705–728 (1951).

(*2*) Wiegenstein, L., and Hain, R. F.: Studies of various tests for malignant neoplastic diseases. II. The Gruskin intradermal test. J. Nat. Cancer Inst. 11:729–732 (1951).

(*3*) ———: Studies of various tests for malignant neoplastic diseases. III. The Hoff-Schwartz intradermal test. J. Nat. Cancer Inst. 11:733–738 (1951).

(*4*) Ellerbrook, L. D., Lippincott, S. W., and Chipps, H. D.: Studies of various tests in malignant neoplastic diseases. IV. The effect of zinc ion upon the serum alkaline phosphatase activity. J. Nat. Cancer Inst. 11:739–756 (1951).

(*5*) Black, M. M., Kleiner, I. S., and Bolker, H.: Changes in the heat coagulation of plasma from cancer patients. Cancer Research 8: 79–82 (1948).

(*6*) Black, M. M., and Speer, F. D.: Chemical tests for malignancy. Am. J. Clin. Path. 20: 446–453 (1950).

(*7*) Black, M. M.: Proceedings of the Conference on Cancer Detection. American Cancer Society, Inc., Portsmouth, N. H., 1949, pp. 138–139, 150–151.

(*8*) Stettner, M. M., Baron, H., Lowenstein, B. E., Greenwald, A. E., Mersheimer, W. L., and Kleiner, I. S.: A clinical evaluation of the Black-Kleiner-Bolker blood test for malignant disease. J. Lab. & Clin. Med. 35: 136–140 (1950).

(*9*) Cullen, G. E., and Van Slyke, D. D.: Determination of the fibrin, globulin, and albumin nitrogen of blood plasma. J. Biol. Chem. 41: 587–597 (1920).

(*10*) Dunn, J. E., and Greenhouse, S. W.: Principles and criteria for the development and evaluation of cancer diagnostic tests. Federal Security Agency, Public Health Service, Publication No. 9, 1950.

APPENDIX

TABLE 4.—*Patients with malignant neoplastic diseases*

No.	Diagnosis	Age	Sex	Metastasis		Result
				Known	No	
1	Basal-cell carcinoma, nose	40	M		x	0. 020
2	Basal-cell carcinoma, ear	67	F		x	. 079
3	Basal-cell carcinoma, scalp	48	F		x	. 037
4	Basal-cell carcinoma, rectum	64	M		x	. 039
5	Squamous-cell carcinoma, face	77	F		x	. 038
6	Squamous-cell carcinoma, nose	78	M	x		. 061
7	Squamous-cell carcinoma, skin	92	M		x	. 068
8	Squamous-cell carcinoma, skin	66	M		x	. 025
9	Squamous-cell carcinoma, penis	78	M		x	. 065
10	Squamous-cell carcinoma, lip	61	M	x		. 054
11	Squamous-cell carcinoma, lip	71	M		x	. 043
12	Squamous-cell carcinoma, lip	58	M		x	. 061
13	Squamous-cell carcinoma, tongue	61	M		x	. 046
14	Squamous-cell carcinoma, larynx	59	F		x	. 037
15	Squamous-cell carcinoma, larynx	53	M		x	. 052
16	Carcinoma, breast	59	F	x		. 048
17	"	54	F	x		. 049
18	"	69	F		x	. 030
19	"	61	F	x		. 041
20	"	43	F		x	. 029
21	"	70	F		x	. 048
22	"	76	F		x	. 065
23	"	78	F		x	. 072
24	"	58	F	x		. 057
25	"	62	F		x	. 080
26	"	63	F		x	. 051
27	"	64	F		x	. 059
28	"	43	F	x		. 043
29	"	64	F		x	. 028
30	"	32	F		x	. 024
31	Sarcoma, breast	78	F	x		. 113
32	Bronchiogenic carcinoma	51	M	x		. 212
33	"	53	F	x		. 043
34	"	49	M	x		. 063
35	"	55	M		x	. 076
36	"	62	M	x		. 068
37	"	71	M	x		. 090
38	"	44	M	x		. 107
39	"	58	M		x	. 142
40	"	61	M	x		. 025
41	"	45	M	x		. 043
42	"	45	M		x	. 173
43	Squamous-cell carcinoma, esophagus	50	M		x	. 089
44	Carcinoma, stomach	76	M	x		. 084
45	"	57	M		x	. 006
46	"	43	F	x		. 061
47	"	73	F	x		. 014
48	"	64	M	x		. 057
49	"	41	F	x		. 226
50	"	62	M	x		. 060
51	"	67	M		x	. 098
52	"	60	M	x		. 060
53	"	70	M	x		. 066
54	Carcinoma, cecum	41	M	x		. 057
55	Carcinoma, colon	76	F	x		. 046
56	"	61	F		x	. 045
57	"	39	F	x		. 050
58	"	83	M		x	. 102
59	"	67	M	x		. 074
60	Carcinoma, rectum, and basal-cell carcinoma, skin	73	M	x		. 045

TABLE 4.—*Patients with malignant neoplastic diseases*—Continued

No.	Diagnosis	Age	Sex	Metastasis		Result
				Known	No	
61	Carcinoma, rectum	81	F		x	0. 041
62	"	67	M		x	. 080
63	"	76	M	x		. 027
64	"	70	M	x		. 055
65	"	72	M		x	. 077
66	"	58	F		x	. 052
67	"	60	M		x	. 085
68	"	65	F		x	. 042
69	"	57	M	x		. 026
70	"	57	M		x	. 090
71	"	66	M		x	. 040
72	"	83	M	x		. 108
73	"	67	F		x	. 042
74	"	70	F		x	. 062
75	"	81	M		x	. 053
76	"	59	M		x	. 061
77	Carcinoma, ampulla of vater	74	M		x	. 098
78	"	36	F		x	. 056
79	Carcinoma, pancreas	61	M	x		. 119
80	"	71	M		x	. 070
81	"	68	M		x	. 074
82	"	70	M		x	. 070
83	Carcinoma, cervix	53	F		x	. 060
84	"	80	F		x	. 078
85	"	54	F	x		. 052
86	"	44	F	x		. 063
87	"	69	F		x	. 055
88	"	76	F		x	. 117
89	"	54	F		x	. 046
90	"	45	F		x	. 012
91	Carcinoma, uterus	59	F		x	. 035
92	"	71	F		x	. 054
93	"	53	F		x	. 041
94	"	60	F		x	. 050
95	"	47	F		x	. 073
96	"	72	F	x		. 064
97	Carcinoma, kidney	55	M		x	. 108
98	"	66	M	x		. 065
99	"	65	M		x	. 104
100	Carcinoma, bladder	71	M		x	. 060
101	"	74	M		x	. 054
102	"	71	M		x	. 072
103	"	44	M		x	. 056
104	"	47	M	x		. 058
105	"	81	M	x		. 109
106	"	63	M	x		. 097
107	Carcinoma, prostate	76	M	x		. 100
108	"	66	M		x	. 066
109	"	80	M		x	. 093
110	"	74	M	x		. 035
111	"	62	M		x	. 095
112	"	59	M	x		. 049
113	"	77	M	x		. 073
114	"	74	M		x	. 075
115	"	81	M		x	. 071
116	"	56	M		x	. 035
117	"	67	M		x	. 142
118	"	67	M		x	. 150
119	Mucoepidermoid carcinoma, parotid	22	F		x	. 034
120	Carcinoma, submaxillary gland	80	M	x		. 046
121	Metastatic carcinoma, type undetermined	48	F	x		. 093
122	"	48	F	x		. 061
123	"	68	F	x		. 062

TABLE 4.—*Patients with malignant neoplastic diseases*—Continued

No.	Diagnosis	Age	Sex	Metastasis		Result
				Known	No	
124	Metastatic carcinoma, type undetermined	73	F	x		0. 041
125	"	41	F	x		. 108
126	"	66	M	x		. 118
127	"	55	F	x		. 032
128	"	73	M	x		. 087
129	"	59	M	x		. 109
130	Melanoma, cheek	70	F	x		. 071
131	Lymphosarcoma	82	F	x		. 034
132	"	73	M	x		. 044
133	"	57	M	x		. 020
134	"	65	M	x		. 103
135	Ewing's sarcoma, sternum	25	F		x	. 048
136	Myelogenous leukemia, chronic	60	M		x	. 025
137	Malignant teratoma, testis	15	M		x	. 047

TABLE 5.—*Patients with benign neoplastic diseases*

No.	Diagnosis	Age	Sex	Result
1	Lipoma	49	F	0. 033
2	"	42	F	. 037
3	"	39	M	. 037
4	"	49	F	. 055
5	"	57	M	. 023
6	"	37	M	. 041
7	Cavernous lymphangioma, peritoneum	53	F	. 046
8	Angioma, brain	22	M	. 068
9	Fibroma, ovary	39	F	. 037
10	Meningioma	50	M	. 062
11	"	62	F	. 048
12	Chondroma, trachea	72	M	. 097
13	Chondro-osteoma, knee	44	F	. 035
14	Enchondroma, phalanx	44	F	. 029
15	Osteoid osteoma, humerus	63	M	. 050
16	Osteoma, skull	36	M	. 030
17	Fibro-adenoma, breast	28	F	. 027
18	"	28	F	. 046
19	"	26	F	. 034
20	"	61	F	. 034
21	Cystadenoma, breast	28	F	. 038
22	Intraductal papilloma, breast	21	F	. 043
23	"	45	F	. 038
24	"	69	F	. 080
25	"	73	F	. 047
26	Papilloma, larynx	36	M	. 046
27	"	25	F	. 034
28	"	51	M	. 021
29	Papilloma, choroid plexus	63	M	. 044
30	Adenoma, thyroid	55	M	. 027
31	"	26	F	. 053
32	"	44	F	. 039
33	Adenoma, rectum	71	M	. 042
34	Leiomyoma, esophagus	28	F	. 045
35	Leiomyoma, uterus	54	F	. 034
36	"	47	F	. 043
37	"	40	F	. 057
38	"	47	F	. 020
39	"	26	F	. 072
40	"	46	F	. 036
41	"	47	F	. 053
42	"	32	F	. 035

TABLE 5.—*Patients with benign neoplastic diseases*—Continued

No.	Diagnosis	Age	Sex	Result
43	Leiomyoma, uterus	40	F	0. 149
44	"	42	F	. 048
45	"	39	F	. 039
46	"	54	F	. 035
47	"	46	F	. 062
48	"	55	F	. 008
49	"	40	F	. 038
50	"	36	F	. 066
51	"	40	F	. 031
52	"	39	F	. 027
53	"	65	F	. 123
54	Leiomyoma, uterus, and cystadenoma, ovary	59	F	. 027

TABLE 6.—*Patients with miscellaneous diseases*

No.	Diagnosis	Age	Sex	Result
1	Cystic disease of breast	48	F	0. 043
2	"	47	F	. 046
3	"	33	F	. 033
4	"	43	F	. 036
5	"	32	F	. 032
6	"	30	F	. 024
7	"	43	F	. 042
8	"	28	F	. 038
9	"	28	F	. 031
10	"	43	F	. 049
11	"	46	F	. 049
12	"	47	F	. 031
13	"	44	F	. 033
14	"	48	F	. 016
15	"	39	F	. 041
16	Cystic disease of breast, and hyperplasia, endometrium	53	F	. 034
17	Intraductal hyperplasia of breast	73	F	. 105
18	Gynecomastia	15	M	. 036
19	Breast abscess	21	F	. 090
20	Osteitis deformans	83	M	. 028
21	Severe cold	43	M	. 028
22	Influenza	42	F	. 082
23	Chronic bronchitis	65	M	. 107
24	"	47	F	. 032
25	Bronchopneumonia	77	M	. 073
26	Bronchiectasis	21	F	. 038
27	Hypertensive cardiovascular disease	46	M	. 052
28	"	47	M	. 020
29	"	54	F	. 038
30	Arteriosclerotic heart disease with angina	77	M	. 028
31	Arteriosclerotic heart disease	69	M	. 070
32	"	74	M	. 095
33	Coronary thrombosis	57	F	. 106
34	"	50	M	. 031
35	Rheumatic fever (active)	55	M	. 074
36	Aneurysm, aorta	61	M	. 075
37	Gangrene, foot	59	F	. 113
38	"	84	F	. 082
39	Internal hemorrhoids	38	F	. 063
40	"	31	F	. 029
41	Cerebral infarction	59	M	. 077
42	Subdural hematoma	27	M	. 035
43	Leukoplakia, lip	62	M	. 039
44	"	60	M	. 068

TABLE 6.—*Patients with miscellaneous diseases*—Continued

No.	Diagnosis	Age	Sex	Result
45	Hyperplasia, thyroid	61	F	0. 025
46	Hyperplasia, lymph node	11	M	. 030
47	Cervical lymphadenitis	28	M	. 035
48	Acute suppurative lymphadenitis	41	F	. 107
49	Chronic tonsillitis	33	F	. 065
50	Gastric ulcer	86	M	. 105
51	"	70	M	. 040
52	"	43	F	. 039
53	"	49	M	. 053
54	"	70	M	. 079
55	"	64	M	. 119
56	Chronic gastritis	68	M	. 068
57	Duodenal ulcer	51	M	. 059
58	"	36	M	. 025
59	"	59	M	. 102
60	"	58	M	. 056
61	"	62	M	. 137
62	"	60	M	. 060
63	Perforated duodenal ulcer	58	F	. 067
64	Diverticulum duodenum	78	F	. 046
65	Periduodenal adhesions	62	M	. 046
66	Diverticulum sigmoid	58	F	. 047
67	"	88	M	. 101
68	Ulcerative colitis	31	M	. 091
69	Amebic colitis	57	F	. 081
70	Regional ileitis	46	F	. 040
71	Acute cholecystitis	55	F	. 084
72	"	79	M	. 042
73	Chronic cholecystitis	69	F	. 040
74	"	56	M	. 096
75	"	80	F	. 098
76	Infectious hepatitis	53	M	. 046
77	"	67	M	. 066
78	Liver abscess	38	M	. 114
79	"	56	M	. 151
80	Steatorrhea	62	M	. 016
81	Acute pyelonephritis	61	M	. 107
82	Pyelonephritis	25	M	. 092
83	Chronic pyelonephritis	62	M	. 115
84	Pyelonephrosis	48	F	. 049
85	Nephroureterolithiasis	39	M	. 128
86	Calculus, ureter	73	M	. 102
87	"	56	M	. 035
88	Calculus, bladder	70	M	. 138
89	"	66	M	. 083
90	Cystitis	62	M	. 041
91	Chronic cystitis	56	M	. 153
92	Cystocele and rectocele	55	F	. 063
93	"	53	F	. 048
94	Rectovaginal fistula	40	F	. 031
95	Benign hypertrophy of prostate	73	M	. 057
96	"	80	M	. 062
97	"	76	M	. 090
98	"	70	M	. 097
99	"	80	M	. 053
100	Benign hypertrophy of prostate, and pyelonephritis	59	M	. 095
101	Benign hypertrophy of prostate	67	M	. 057
102	"	67	M	. 021
103	"	68	M	. 067
104	"	85	M	. 097
105	"	74	M	. 143
106	"	70	M	. 049
107	Chronic prostatitis	60	M	. 035
108	Chronic epididymitis	39	M	. 072

TABLE 6.—*Patients with miscellaneous diseases*—Continued

No.	Diagnosis	Age	Sex	Result
109	Tubo-ovarian abscess	16	F	0. 055
110	"	30	F	. 146
111	Chronic cervicitis	19	F	. 051
112	Chronic cervicitis, and prolapse, uterus	42	F	. 026
113	Chronic cervicitis	18	F	. 018
114	"	63	F	. 065
115	"	73	F	. 091
116	"	66	F	. 044
117	"	28	F	. 020
118	"	48	F	. 047
119	Chronic endometritis	48	F	. 029
120	"	36	F	. 014
121	Atrophy, endometrium	72	F	. 010
122	Hyperplasia, endometrium	34	F	. 049
123	"	30	F	. 054
124	"	40	F	. 026
125	"	42	F	. 029
126	"	23	F	. 059
127	"	24	F	. 020
128	"	43	F	. 042
129	"	37	F	. 060
130	"	26	F	. 040
131	"	56	F	. 019
132	Adenomyosis, uterus	40	F	. 027
133	"	44	F	. 023
134	Retention of secundines	36	F	. 026
135	Hyperthyroidism	60	F	. 071
136	Diabetes mellitus	55	F	. 061
137	Meningeal adhesions due to trauma	38	M	. 054
138	Epidural abscess	27	M	. 040
139	Ganglion, ankle	25	M	. 030
140	Synovial cyst, foot	33	M	. 036
141	Dacrocystitis	59	F	. 044
142	Retroperitoneal tuberculosis	47	M	. 056
143	Myositis ossificans	17	M	. 029
144	"	32	M	. 067

929548—51——12

THE MEASUREMENT OF PROLIFERATION IN TISSUE CULTURES BY ENUMERATION OF CELL NUCLEI [1]

KATHERINE K. SANFORD, WILTON R. EARLE, VIRGINIA J. EVANS, HELEN K. WALTZ, and JOHN E. SHANNON,[2]
National Cancer Institute, National Institutes of Health, U. S. Public Health Service, Bethesda, Md.

INTRODUCTION

One problem in quantitative work with tissue cultures has been the measurement of culture growth. Although numerous methods of measurement have been proposed and used, there has been no accurate nor easily applied procedure for estimating proliferation in a culture over a wide range of experimental conditions. So long as the conventional tissue cultures were in use in which the cells grew from a central explant embedded in a plasma clot, any accurate enumeration of cells or cell nuclei appeared impractical, but with the development of methods (*1*) for growing large numbers of replicate cultures on surface substrates such as cellophane, a new approach to the problem seemed possible.

A procedure has been developed in this study by which cell nuclei may be quantitatively removed from the culture substrate, stained, suspended in a known volume of fluid, and the number of nuclei present in an aliquot of the suspension enumerated. The number of cell nuclei may thus be determined in replicate cultures randomly selected at intervals during the period of study. By such a sampling procedure changes in numbers of the cell nuclei in cultures under different experimental conditions may be followed. The rate of proliferation as determined by this method is the algebraic sum of the rate of nuclear division and nuclear disintegration within the culture.

This paper presents the method developed particularly for the strain L mouse cells but applicable to other cell types, and also presents the experimental development and testing of this procedure.

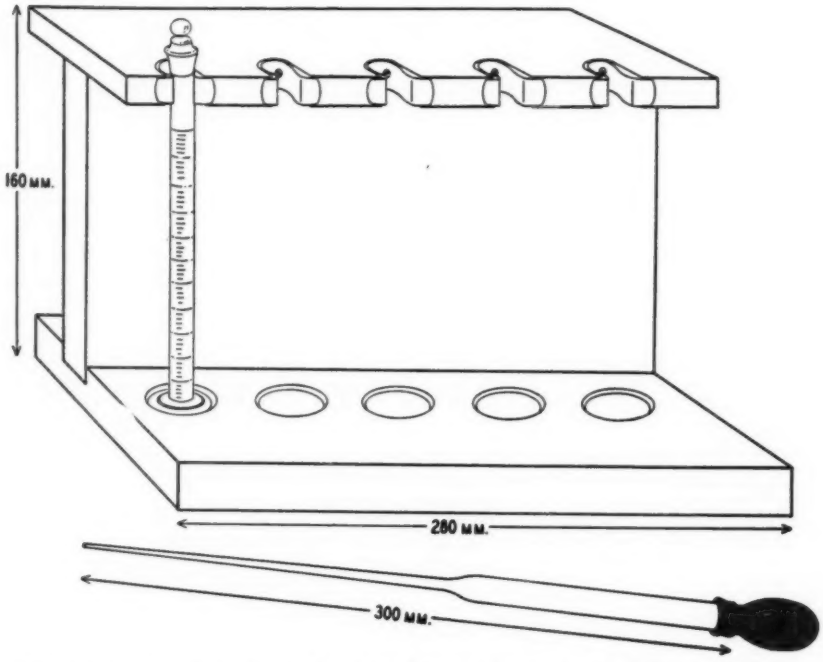
MATERIALS

The solutions and equipment used in the enumeration procedure were as follows:

Citric acid, 0.1 M, to which a few crystals of thymol were added to prevent mold growth.

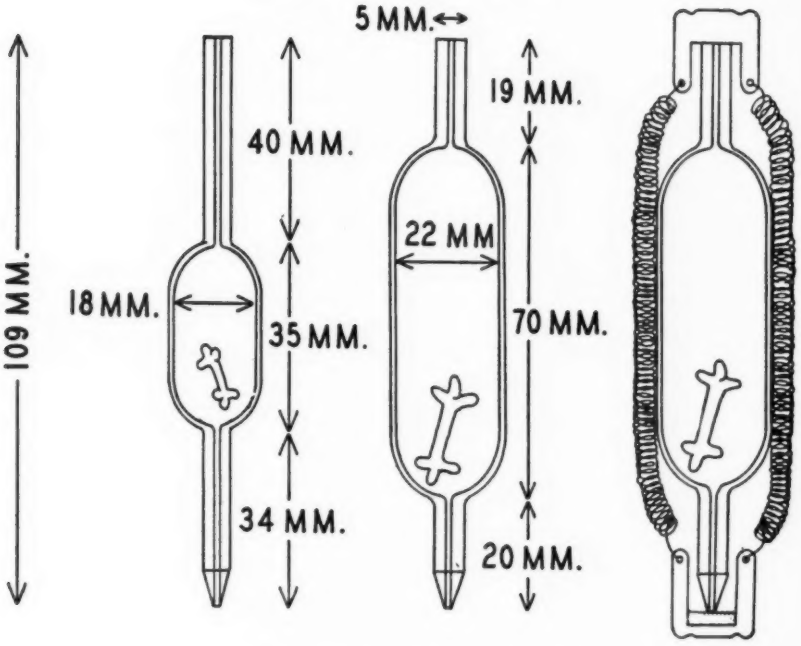
[1] Received for publication September 27, 1950.
[2] The authors wish to thank Mr. Schneiderman of the Biometrics Section of this Institute for his guidance in the statistical developments of this study.

Methocel,[3] 4,000 cps; 0.5 percent dissolved in 0.1 M citric acid.
Crystal violet, 0.1 percent dissolved in 0.1 M citric acid.
Centrifuge, International,[4] size 2, fitted with indicating tachometer, head #277 carrying 250-ml. tubes #384, and head #240 carrying 100-ml. tubes #340.
Graduated cylinders, Pyrex, 10 ml. with S. T. glass stopper and with base ground to slip into 100-ml. centrifuge tubes.
Rack for graduated cylinders, shown in text-figure 1.
Long-tip pipette, shown in text-figure 1.
Beakers, Pyrex, 30 ml.
Counting pipettes, special design shown in text-figure 2, of approximately 5-ml. and 15-ml. capacity, each fitted with sealing device.
Blood-cell counting-pipette rotor, Bryan-Garrey.[5]
Hemocytometers,[6] bright-line, double improved Neubauer ruling with 0.4-mm. thickness cover glasses.
Binocular microscope equipped with 8-mm. achromatic objective and with Bausch and Lomb ×15 wide-field oculars.
Hand counters (tally register), Veeder.



TEXT-FIGURE 1.—Rack for graduated cylinders, and long-tip pipette used in removal of supernatant fluids.

[3] Dow Chemical Company, Midland, Michigan.
[4] International Equipment Company, Boston, Massachusetts.
[5] A. S. Aloe Company, St. Louis 3, Missouri.
[6] American Optical Company, Buffalo 15, New York.



COUNTING PIPETTES AND SEALING DEVICE

TEXT-FIGURE 2.—Counting pipettes and sealing device. The sealing device consists of 2 brass cups, fitted with rubber cushions, and connected by springs of gauge #8 piano wire.

PROCEDURE

Approximately 14 ml. of 0.1 M citric acid, warmed to 37° C., was added to each culture growing in 2 ml. of culture fluid in a T–12 flask (*1*). The culture was incubated at 37° C. for 1 hour, then shaken vigorously by hand. The nuclei were thrown down by centrifugation for 20 minutes at 1,100 r. p. m. After approximately 12 ml. of supernatant was withdrawn by a long-tip pipette and discarded, 1 ml. of 0.1 percent crystal violet was added to each flask. The flask was then shaken by hand, and the contents transferred quantitatively, with 3 to 4 citric-acid washes, to a 10-ml. graduated cylinder. Before the transfer the outer lip of each flask was coated with yellow petrolatum to prevent dripping and consequent loss of fluid in transferring the contents. After the cylinders were centrifuged at 1,800 r. p. m. for 20 minutes, the supernatant was withdrawn by a long-tip pipette so that a volume equal to one half the final volume was left in each cylinder. To this volume an equal volume of 0.5 percent methocel was added. The final volume of suspension was roughly approximated to yield about 300,000 nuclei per ml. The graduated cylinder was stoppered and shaken to disperse the packed nuclei. While the nuclei

were still uniformly dispersed, the suspension was transferred immediately to a 30-ml. beaker, which was kept agitated during the withdrawal of the contents into the special counting pipette. If the counting was to be delayed, the pipettes were sealed and stored at 5° C. At the time of counting, the pipettes were clamped in the Bryan-Garrey rotor and rotated for at least 15 minutes to establish and maintain a uniform dispersion of nuclei before samples were taken. Before charging the hemocytometer chamber, the contents of the tip of the counting pipette were discarded. A sample of the suspension was then discharged into one chamber of a hemocytometer positioned on the microscope stage.

Each suspension of nuclei was sampled and counted by three operators. Each operator filled one hemocytometer chamber, and counted the nuclei in the 9 1-mm. squares or hemocytometer units. Since the nuclei in a total of 27 squares were counted by the three operators, a volume of 2.7 cu. mm. was counted on each suspension. The number of nuclei per culture was calculated from the following formula:

$$\frac{\text{Total number of nuclei counted} \times \text{Total volume of suspension in cu. mm.}}{\text{Volume of sample counted in cu. mm.}} = \text{Number of nuclei per culture}$$

Example: If 3 operators counted a total of 832 nuclei in 27 hemocytometer squares, or a volume of 2.7 cu. mm. of a suspension which had been diluted to 10 ml. or 10,000 cu. mm., the number of nuclei per culture would be calculated as follows:

$$\frac{832 \times 10{,}000}{2.7} = 308{,}148 \text{ nuclei per culture}$$

EXPERIMENTAL RESULTS

Action of Citric-Acid Solution on Cells

Although living cells could not be completely removed from a glass or cellophane substrate by shaking the flask, the nuclei of cells fixed with citric acid could be completely removed as shown by microscopic examination of the substrate.

To determine whether any cell nuclei were disrupted by this treatment with citric acid, young cultures of cells growing under perforated cellophane were photographed before and after citric-acid treatment, and the photomicrographs compared. The examination of 850 individual nuclei in these photomicrographs revealed no disintegration of nuclei. Cells photographed before and after 1 hour of citric-acid treatment are shown in figures 1 and 2.

Although the cytoplasm was not disintegrated by the citric acid, it became so fragile that shaking and washing usually stripped it from the nucleus. Any cytoplasmic tags left on the nuclei did not interfere with counting of the diluted suspension as long as these remnants did not cause clumping of nuclei. A slightly shaken preparation and a shaken and washed preparation of unstained nuclei in citric acid are shown in figures 3 and 4, respectively.

Effect of Age of Culture

In young, rapidly growing cultures the citric acid-crystal violet diluting solution clearly fixed and sharply stained the chromatin of the nucleus. Cells in mitosis were so clearly fixed that the mitotic figures could be enumerated differentially and each stage of mitosis recognized. Several mitotic figures are shown in figures 3, 4, and 8.

In older, partially necrotic cultures staining of the necrotic cell nuclei was less sharp, while the stained nuclei of cells undergoing severe degeneration became achromatic and ultimately disintegrated to unrecognizable debris. The nuclei of normal healthy cells and the nuclei of cells in early stages of necrosis could be differentially enumerated, but the nuclei of cells in later stages of necrosis could not be counted because of their disintegration. The effect of citric acid on an almost completely necrotic culture is illustrated in figures 5 and 6.

Factors Affecting the Preparation of Suspensions of Nuclei

pH of the citric-acid solution.—A pH satisfactory for isolation of nuclei resulted from the use of at least 7 volumes of 0.1 M citric acid solution for each volume of the heavily buffered culture medium used (*1*). The pH of such mixtures of the acid with fresh culture fluid, or of the acid with old culture fluid in which cells had been growing for 48 hours, ranged from 2.26 to 2.30 as measured by glass electrode. At this pH adequate isolation of nuclei to yield a homogeneous suspension was obtained after 1 hour of incubation at 37° C. and subsequent centrifugations and washings.

The pH of the final solution appeared to affect the sharpness of image of the cell nucleus, since after washing of a citric-acid-fixed culture with a nearly saturated solution of Na_2CO_3, the sharp image of the nuclear membrane and chromatin was lost. A subsequent wash in citric acid restored some of this sharpness of image as illustrated in figures 7 through 10.

Washing of the cells.—With old cultures undergoing disintegration, it was often advantageous to wash the cells several times with citric acid to eliminate some of the debris from the cultures. If the cells were suspended in a high protein medium such as 6 percent albumin saline, a wash of balanced saline was necessary, before addition of the citric acid, to avoid a precipitate.

Osmotic pressure of the citric-acid solution.—Both hypotonic citric-acid solution and citric-acid solution made isotonic by the addition of NaCl were tested. The osmotic pressure of the isotonic solution was established by freezing-point determination. Treatment of the cells with the hypotonic solution yielded better isolation of cell nuclei than treatment with the isotonic solution.

Temperature and duration of citric-acid treatment.—An effort was made to shorten the nuclei-isolation procedure by shortening the period of citric-acid treatment and by raising the temperature of treatment. Both 20- and 30-minute periods of citric-acid treatment at 37° C. were tried and were found to be inadequate in that the nuclei even after vigorous shaking were incompletely isolated and clumped. When the cells were

given a preliminary wash with saline or citric acid before the incubation, this shorter treatment was adequate; but such a procedure involving the additional washing and centrifugation was as time consuming as the original method employing 1 hour of incubation. Two higher temperatures of incubation, 50° C. and 75° C., were tried. There appeared to be no acceleration of citric-acid action at either of these temperatures.

Type of acid used.—Since previous experiments suggested that decreased pH and hypotonicity of solution played a definite role in the action of citric acid on cells, solutions of lactic and maleic acid having the same osmotic pressure—measured by freezing point depression—as 0.1 M citric acid were tested. These acids have dissociation constants for the first hydrogen of 1.38×10^{-4} and 1.5×10^{-2}, respectively, as compared with the dissociation constant of 8.4×10^{-4} for citric acid. The lactic-acid solution was found to be as effective as citric acid in isolating the nuclei. With the stronger acid, maleic, however, the isolation of nuclei was inadequate, and cells were clumped. Even when the cell suspension in culture fluid was given an initial saline wash, the maleic-acid treatment failed to yield isolated nuclei.

Stability of Suspensions of Fixed Nuclei

The stability of nuclei after fixation was determined by comparing the numbers of nuclei in suspensions counted immediately after preparation with the numbers of nuclei in the same suspensions counted after 7 days' storage at 5° C. The duplicate counts are presented in table 1. No significant difference between the 1- and 7-day counts was found. These results indicate that the nuclei were stable for at least 7 days when stored at 5° C. A group of cultures could thus be fixed, and their nuclei enumerated, as desired, within the following 7-day period.

Table 1.—*Stability of suspensions of fixed nuclei at 5° C.*

Suspension No.	Number of nuclei counted		Difference
	First day	Seventh day	
1	1, 726	1, 760	+34
2	1, 756	1, 784	+28
3	1, 566	1, 883	+317
4	1, 841	1, 839	−2
5	1, 836	1, 835	−1
6	1, 437	1, 460	+23
7	748	843	+95
8	732	798	+66
9	738	650	−88
10	759	722	−37
11	755	842	+87
12	772	732	−40
13	851	764	−87
14	868	813	−55
15	790	850	+60
Average difference			[1] 26. 67

[1] Standard error of average difference = 25.65; t value $\left(\frac{\text{average difference}}{\text{standard error of average difference}}\right)$ = 1.04; p value = 30 to 40 percent.

Influence of Suspending Agent on Uniformity of Counts

The influence of suspending agents on the uniformity of distribution of nuclei in the counting chamber was also determined. The three agents in the final concentration tested were: 3 percent gum ghatti,[7] 0.25 percent methocel of 4,000-cps. viscosity, and 0.25 percent methocel of 1,500-cps. viscosity. The results of this study are presented in table 2, where the influence of each of these agents on the uniformity of count is compared with the results when no suspending agent was used.

Table 2—*Influence of suspending agents on uniformity of count*

Suspending agent	Number of hemocytometer units counted	Average number of nuclei per hemocytometer unit	Weighted average of F values [1]	F ratio (Comparison with control) [1]
None (control)	250	44. 77	2. 10	
3 percent gum ghatti	125	44. 76	1. 55	1.358 (probably significant).
0.25 percent methocel (4,000 cps.)	370	47. 11	1. 41	1.485 (highly significant).
0.25 percent methocel (1,500 cps.)	367	42. 74	1. 74	1.207 (possibly significant).

[1] F value $= \frac{\text{computed variance}}{\text{theoretical variance}}$. The theoretical variance was considered the average count per hemocytometer unit (*2*). The F values computed in this table are approximations to Snedecor's F. The tabled F distribution can be used when entered with $(n-1)$, $(2\times n)$ degrees of freedom. As shown by Mantel and Schneiderman (personal communication), this is a conservative approximation to F. Weighted average of the F values $= \frac{\Sigma(n\text{-}1)\,F}{\Sigma(n\text{-}1)}$ where n = number of hemocytometer units counted.

The results indicate that a final concentration of 0.25 percent methocel of 4,000-cps. viscosity significantly increased the uniformity of distribution of nuclei in the suspension. This concentration of 4,000-cps. viscosity methocel was significantly more effective in distributing the nuclei than methocel of 1,500-cps. viscosity. The methocel preparations were more satisfactory for microscopic observation and counting than the gum ghatti preparations, since in the gum ghatti fine particulate matter was present that could not be removed.

Influence of Depth of Hemocytometer Chamber on Accuracy of Count

Suspensions of nuclei were counted in hemocytometers having chamber depths of 0.1, 0.2, and 0.4 mm.[8] The deep chambers were tested particularly for use with dilute suspensions of fewer than 250,000 nuclei per ml. The data from this study are presented in table 3.

Although the counts from the 0.1- and 0.2-mm.-deep chambers did not differ significantly from the theoretical or Poisson distribution (*2*), the counts from the 0.4-mm.-deep chambers did differ significantly. The difference in uniformity of distribution between the 0.1- and the 0.4-mm.-deep chambers was highly significant. This difference might have been due, however, to the difference in the average number of nuclei counted

[7] Prepared according to Hawk, P. B., Osler, B. L., and Summerson, W. H.: Practical Physiological Chemistry, Blakiston Co., Philadelphia, 12th Ed., 1947, p. 1227.

[8] The 0.2 and 0.4 mm deep chambers were specially designed for this research by C. A. Hausser and Son, 6040 Belfield Ave., Philadelphia 44, Pa.

TABLE 3—*Influence of depth of hemocytometer chamber on accuracy of count*

Chamber depth in millimeter	Index of uniformity [2]	Average number of nuclei per hemocytometer unit	Number of hemocytometer units	*F* ratio (comparison with 0.1-millimeter slide)
0.1	1.56	19.8	80	
	.82	24.2	80	
	1.12	21.9	80	
0.2	1.15	37.7	80	1.048 (not significant).
	.84	46.4	80	
	1.35	40.2	80	
0.4	5.94	68.6	80	3.265 (highly significant).
	2.63	90.7	80	
	2.61	80.0	65	2.099 [3] (highly significant).
0.1 [1]	2.59	93.9	20	
	1.45	93.9	30	
	1.59	66.0	20	

[1] Data drawn from different experiments to provide comparison with 0.4-mm. deep chamber for approximately the same average numbers of nuclei per hemocytometer unit.
[2] Index of uniformity—*F* value as in table 2.
[3] Comparison with 0.1-mm. deep chamber having approximately the same average number of nuclei per hemocytometer unit.

per hemocytometer unit in the two types of chamber, since when large numbers of nuclei were counted in each hemocytometer unit, more errors in counting were found to occur. A set of data from 0.1-mm. chamber counts having the same average number of nuclei per hemocytometer unit was, therefore, compared with the data from the 0.4-mm. chamber counts. There were still highly significant differences between the two groups of data in the uniformity of nuclei distribution. The 0.1-mm. chamber gave considerably more uniform counts. These results indicate that the use of as deep a chamber as 0.4 mm. may impair the accuracy of the counts. Much of this difficulty in counting nuclei in the deep chamber appeared to result from the continuous focusing necessary to detect nuclei in the deeper layers of fluid. Since even with the 0.2-mm. chamber considerable focusing was necessary, the 0.1-mm. chamber was selected as the most accurate and practical for routine use.

EVALUATION OF ACCURACY OF THE COUNT PROCEDURE

To determine the number of nuclei to be counted to give a desired level of accuracy, the following formula (*3*) was used:

$$\text{Percentage error} = \frac{k\,C\,v}{\sqrt{n}}$$

where k=a constant defining the percentage of times the desired accuracy or better is to be attained (*i e.*, k=1.96 when the desired level of accuracy is to be attained 95 percent or more of the time). Cv=coefficient of variation or $\frac{s}{m}$ where s=computed standard deviation and m=average

count per hemocytometer unit. n=number of hemocytometer units or 1-mm. square areas counted.

It has been shown (*2*) that counting particles in a hemocytometer leads to a Poisson distribution in which $s=\sqrt{m}$ or $Cv=\frac{1}{\sqrt{m}}$.

The level of accuracy arbitrarily set for the present procedure was 90 percent, 95 percent of the time.

By substitution in the above formula, $0.10=\frac{1.96}{\sqrt{n}}\times\frac{1}{\sqrt{m}}$, it is found that $n=\frac{384}{m}$. In preliminary experiments, the actual variance, s^2, among the units was twice the expected theoretical variance; thus the square of the coefficient of variation was doubled, and n became two times 384 or $m\times n=768$ nuclei.

The number of nuclei to be counted to attain an accuracy of 90 percent, 95 percent of the time, thus lay between 750 and 800.

An examination of the nuclear distribution in the hemocytometer indicated that a total of 30 to 45 nuclei distributed over each 1-mm. square area or hemocytometer unit was the most advantageous number for accurate counting. When higher numbers of nuclei were distributed in each hemocytometer unit, inaccuracies were introduced in counting that tended to raise the variance. When smaller numbers of nuclei were distributed in each unit, more units had to be counted. For these reasons, a total of 30 to 45 nuclei per hemocytometer unit was considered as the most workable range for accurate and rapid counting.

As the counting technique was improved, operator differences became insignificant and accuracy increased. In the last 16 counts in which 700 to 800 nuclei were enumerated from 11 different suspensions prepared and counted according to the procedure outlined in this paper, the errors ranged from 5.6 to 10.5 percent, with all errors but 1 less than 8.7 percent. These data are presented in table 4 together with 6 additional counts of suspensions that had a few microscopic clumps of nuclei. The slightly higher errors of these latter 6 counts illustrate the tendency for clumping to interfere with the accuracy of counting.

In addition to the errors of counting, errors might be introduced during the processing of the living cells. The errors of processing may be estimated from determination of total-percentage error among replicate cultures counted at the time of planting. The total error, including differences among the flasks and errors from the processing and counting of the suspensions, was less than 10 percent in 9 experiments, in which 3 to 6 flasks were enumerated in each experiment (*1*). Since the major part of the total error can be accounted for by the counting error and differences among flasks, the processing error appears to be negligible.

TABLE 4.—*Accuracy of counting*

Pipette No.	Suspension No.	Number of nuclei counted	Average number of nuclei per hemocytometer unit	Percentage error[1] (95 percent limits)
1	1	716	26. 5	8. 1
2	1	731	27. 1	6. 1
3	1	702	26. 0	10. 5
4	2	737	27. 3	8. 3
5	3	782	35. 6	6. 6
6	3	772	42. 9	7. 3
7	3	727	40. 4	7. 2
8	4	771	35. 1	7. 4
9	4	771	35. 1	8. 3
10	5	800	27. 6	6. 4
11	6	782	29. 0	5. 6
12	7	743	23. 2	7. 4
13	8	764	28. 3	8. 7
14	9	766	47. 9	7. 6
15	10	782	24. 4	7. 3
16	11	790	34. 4	8. 0
17	12	789	25. 5	[2] 8. 7
18	13	727	22. 7	[2] 7. 9
19	14	782	55. 9	[2] 10. 2
20	15	785	60. 4	[2] 9. 4
21	16	731	22. 8	[2] 9. 2
22	17	772	42. 9	[2] 11. 1

[1] Percentage error $=\frac{kCv}{\sqrt{n}}$ where $k=1.96$, since the level of accuracy was computed for 95 percent of the time (95 percent limits); Cv=coefficient of variation; n=number of hemocytometer units counted.
[2] Small clumps of nuclei present in the suspension.

DISCUSSION

Methods used as quantitative measures of "growth" in tissue culture have been considered in a review by Cunningham and Kirk (*4*) which reveals many of the inadequacies of the procedures currently used. The present method provides an accurate measure of the numbers of nuclei present in a culture, and as such, may be used as a tool in the study of proliferation in cultures under varying experimental conditions. The method has an advantage over any chemical determination in that the nuclei of healthy cells and of cells in early stages of necrosis may be distinguished and differentially enumerated. The different stages of mitosis may also be distinguished.

Although this method has been worked out in detail for one cell strain, the strain L mouse cells, the method should be applicable to other cell types. In preliminary trials, the method was satisfactory for the isolation of nuclei from cultured liver cells, but could not be used for a strain of chicken-heart fibroblasts, which had laid down a heavy fibrous matrix.

In adapting nuclei-isolation procedures such as those of Dounce (*5*) and Marshak (*7*) for this quantitative method, certain modifications were introduced. In their procedures, tissues are ground either in a mortar, Waring blendor, or other high-speed mixer. Such grinding of the tissues damages or destroys a certain fraction of the nuclei (*5*). This step was, therefore, eliminated in the present method in which the nuclei in all living cells must be maintained intact.

Both in the present study and in the study by Dounce (*6*), a hypotonic solution of citric acid was more effective than a solution of higher osmotic pressure in isolating the cell nuclei. These results suggested that the isolation might possibly depend on a rupturing of cells produced by the hypotonic solution. If the cells were ruptured by the hypotonic solution, some nuclei might also be ruptured and destroyed when the citric acid first came in contact with the cells. To test this possibility cells were therefore studied by photomicrography before and after treatment with citric acid. This study revealed no instance of nuclear disruption or disintegration when citric acid was added. No obvious swelling or rupturing of cells occurred even after a period of 3 days of citric-acid treatment. Although the hypotonicity of citric-acid solution was apparently essential for nuclei isolation, the hypotonicity was not sufficient to cause recognizable swelling or rupturing of either cells or nuclei. The citric acid appeared to harden the nuclei and increase the fragility of the cytoplasm, so that subsequent shaking, centrifugation, and washing stripped the cytoplasm from the nucleus.

To determine whether fixation of the cell nucleus by the citric acid was due to the specific action of the citrate-ion, or to the hydrogen-ion concentration, Marshak (*7*) treated liver and tumor tissues with 5 percent acetic acid and 5 percent boric acid in place of citric acid. Nuclei could not be isolated after treatment with boric acid; but after treatment with acetic acid the nuclei could be isolated as readily as after citric-acid treatment. At a pH of 6.0, free nuclei could be obtained, but such nuclei were optically homogeneous. Nuclei showed granularity only when in a solution of a pH level of 5.0 or lower. The conclusion was drawn that evidently the separation of the nucleus from the cytoplasm and the coagulation of the nucleus are due to an increase in hydrogen-ion concentration and not to the action of a particular anion.

In the present study the two acids, lactic and maleic, were tested in place of citric acid in an attempt to find an acid that might act more rapidly than citric acid. Although lactic acid, which has only a slightly greater dissociation constant than citric acid, was effective, the more highly dissociated maleic acid produced severe clumping of cells and inadequate fixation of nuclei for subsequent isolation, even after a preliminary saline washing of the cell suspension.

The results of the present study and the observations of Marshak (*7*) suggest that citric acid may be replaced by acids of similar dissociation constant, such as acetic and lactic, whereas acids of lower or higher dissociation, such as boric and maleic, respectively, are ineffective. Both the fixation of nuclei for subsequent isolation and the sharpness of image of the nuclear membrane and chromatin appear to depend on the pH of the suspending fluid.

Each step in the process of isolating cell nuclei and finally counting the suspensions may introduce some degree of error. Possible sources of error in this procedure may be listed as follows: 1) Loss of cells or nuclei in the supernatant fluids removed. 2) Loss of nuclei in the transfer of

suspensions from flasks to graduated cylinders. 3) Inaccuracies of measurement in the graduated cylinders. 4) Incomplete dispersion of nuclei during the transfer of suspensions from graduated cylinder to beaker or from beaker to pipette. 5) Sampling errors arising from incomplete dispersion of nuclei in the pipette at the time of charging the counting chamber. 6) Faulty technique in filling of the counting chamber. 7) Inaccuracies in counting. 8) Leakage from inadequately sealed pipettes.

Precautionary measures were introduced in the enumeration procedure to prevent certain of these errors, since in any quantitative technique accuracy is necessary at each stage of the method. The long-tip pipette and stationary racks for graduated cylinders facilitated the removal of supernatant fluids without serious agitation of packed nuclei. The application of a thin coating of petrolatum on the outer lip of the flask helped in preventing loss of drops of suspension in the transfers of suspensions from flasks to graduated cylinders. To prevent rapid sedimentation of particles and to aid in maintaining uniform suspensions, the suspending agent, methocel having a viscosity of 4,000 cps. was added to each suspension. After considerable practice, each operator developed skill in charging the hemocytometer chamber. To prevent inaccuracies of counting, mechanical counters were used. An optical system, employing $\times 15$ wide-field oculars with an 8-mm. achromatic objective gave a broad, flat field facilitating microscopic observation; and the crystal-violet staining solution further sharpened and defined the nuclear image. An adequate pipette-sealing device was developed that prevented accidental leakage of pipettes.

With experienced operators, most of these eight possible sources of error could be eliminated. Of the eight sources, the incomplete dispersion of nuclei, particularly at the time of "sampling" or counting, appeared to be the most serious. If suspensions of nuclei contained even minute clumps of nuclei, such clumps tended to reduce the counting accuracy. Clumping was most frequently observed in suspensions prepared from densely packed cultures 4 to 5 weeks of age or from cultures of cells suspended in fluid of high-protein concentration. For such preparations, several preliminary washes with saline were found to be essential to prevent precipitation in the medium when the citric acid was added.

In applying the enumeration procedure to the determination of proliferation in replicate cultures, groups of cultures could be processed and the nuclei enumerated at intervals during the growth period. Within each group of cultures grown under the same environmental conditions, the total variance observed in the data consisted of the biologic variation between cultures or between flasks and the variations resulting from errors in processing and enumerating the nuclei suspensions. The total error could thus be separated into two components (*8*) as follows:

Total variance or $s^2=\frac{s_c^2}{n_c}+\frac{s_t^2}{n_t}$ in which: s_c^2=the count variance; n_c=the

number of nuclei counted; s_f^2=the flask variance; n_f=the number of flasks counted.

The first component, the error due to the count procedure, could be reduced by increasing n_c, or the number of nuclei counted. Although with the count procedure, an accuracy of 90 percent or better, 95 percent of the time, was attained by counting 27 hemocytometer units or approximately 800 nuclei, higher levels of accuracy could be attained by counting additional hemocytometer units or numbers of nuclei. For instance, to attain an accuracy of 95 percent, 95 percent of the time, 106 hemocytometer units or approximately 3,200 nuclei could be counted. Since additional counting was both laborious and time consuming, the 90-percent level of accuracy 95 percent of the time was selected as providing a satisfactory, workable level of accuracy.

The second component, the error due to differences between flasks or cultures, could likewise be reduced by increasing the total number of flasks used for a determination. In preliminary tests a total of 3 flasks was used for each determination. Increases in the numbers of flasks to be processed and counted slowed the determinations. With the method outlined, one trained operator could prepare about 36 cultures for counting during one working day, while 1 to 2 days were needed for the counts.

By minor changes, however, the procedure may be altered to allow the rapid determination of a larger number of flasks in each experimental group. If, for example, 90 replicate cultures were planted in 1 experiment and were split into sets of 9 cultures each, for testing 10 different variables, the suspensions from each of 9 cultures might be pooled into 3 lots of 3 cultures each, and the nuclei in each lot separately enumerated. With this modification, the error due to differences in flasks could be reduced; in addition, 1 trained operator should be able to complete the preparation and counting of the 90 cultures testing 10 variables at the assigned level of accuracy in about 2 or 3 days. This altered procedure thus appears feasible for large-scale experiments.

SUMMARY AND CONCLUSIONS

A procedure has been developed by which the nuclei from cultures grown on surface substrates such as cellophane or glass, may be quantitatively removed from the substrate and enumerated. Each culture was treated with 0.1 M citric-acid solution. The cell nuclei werei solated by at least two centrifugations at 1,100 and 1,800 r.p.m. for 20-minute periods, and were resuspended in a known volume of 0.1 M citric acid containing a final concentration of 0.25 percent methocel of 4,000-cps. viscosity and 1 ml. of 0.1 percent crystal-violet solution. The nuclei suspension was drawn into a specially designed pipette for use in a Bryan-Garrey blood-cell-counting rotor. After uniform dispersion of the nuclei in the pipette, the nuclei were enumerated in a standard hemocytometer. In such a preparation, nuclei in mitosis, normal resting nuclei, and the nuclei of cells in early stages of necrosis could be distinguished. With this method, a counting accuracy of 90 percent, or better, 95 percent of the time was

attained. This procedure used in conjunction with the procedure for preparing and handling large numbers of replicate cultures provides a technique for following, accurately and easily, changes in the number of nuclei in cultures under experimental study. With the slight modification suggested of pooling cultures, one trained worker should be able to prepare and count 90 cultures testing 10 variables at the assigned level of accuracy in about 2 or 3 days.

REFERENCES

(1) Evans, V. J., Earle, W. R., Sanford, K., Shannon, J., and Waltz, H.: The preparation and handling of replicate tissue cultures for quantitative studies. J. Nat. Cancer Inst. In press.

(2) Fisher, R. A.: Statistical methods for research workers. Oliver and Boyd, Edinburgh, 8th ed., p. 54 (1941).

(3) Snedecor, G. W.: Statistical methods. Iowa State Collegiate Press, 4th ed., p. 457 (1946).

(4) Cunningham, B., and Kirk, P. L.: Measure of "growth" in tissue culture. J. Cell. & Comp. Physiol. 20: 343–358 (1942).

(5) Dounce, A. L.: The preparation of isolated cell nuclei of rat liver. Science 99: 512–513 (1944).

(6) ———: Enzyme studies on isolated cell nuclei of rat liver. J. Biol. Chem. 147: 685–698 (1943).

(7) Marshak, A.: P_{32} uptake by nuclei. J. Gen. Physiol. 25: 275–291 (1941).

(8) Berkson, J., Magath, T. B., and Hurn, M.: Error of estimate of blood cell count as made with the hemocytometer. Am. J. Physiol. 128: 309–323 (1939).

Plate 78

Figure 1.—Strain L mouse cells growing under perforated cellophane in D–3.5 Carrel flask. × 400.

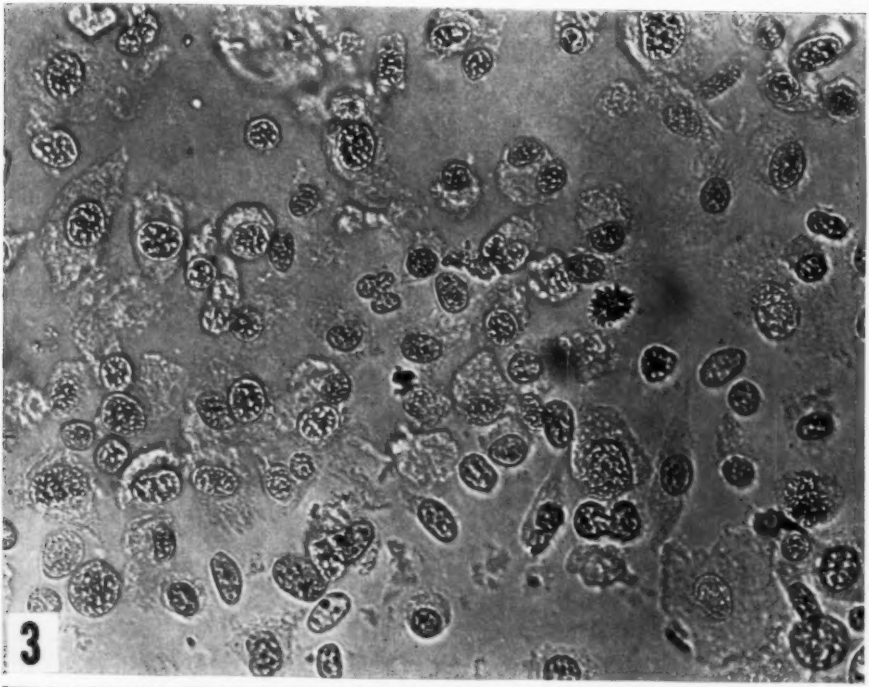
Figure 2.—Cells of the same field as figure 1 after 1 hour's treatment with 0.1M citric acid. × 400.

929584—51——13

PLATE 79

FIGURE 3.—Cells of the culture of figures 1 and 2 after citric-acid treatment and after shaking the flask. Note the clearly defined mitotic figure. × 400.

FIGURE 4.—A washed, unstained suspension of nuclei of strain L mouse cells fixed with citric acid. Note the mitotic figures. This photograph is slightly out of focus to sharpen the nuclear outline. × 400.


3

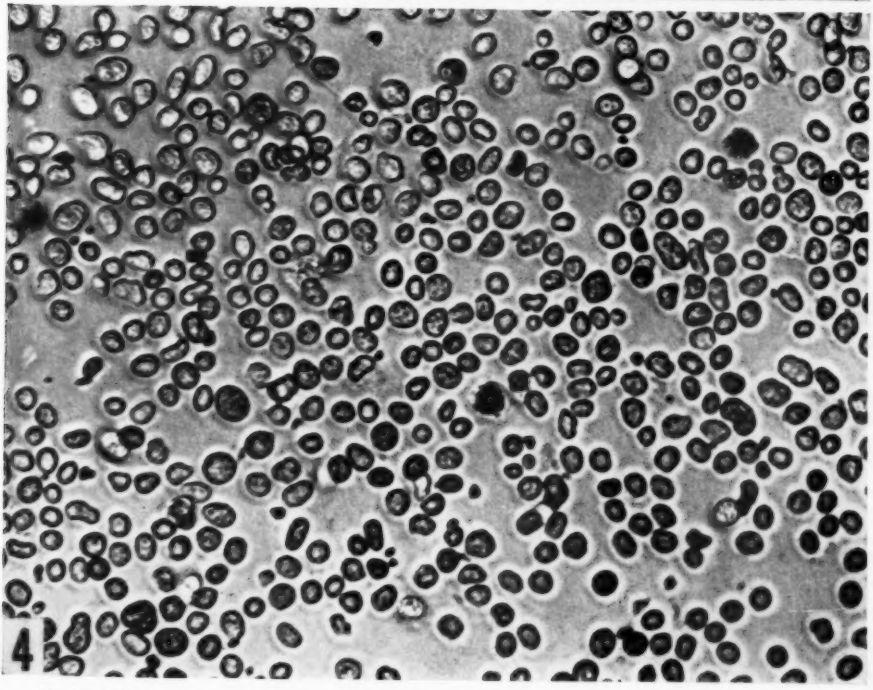
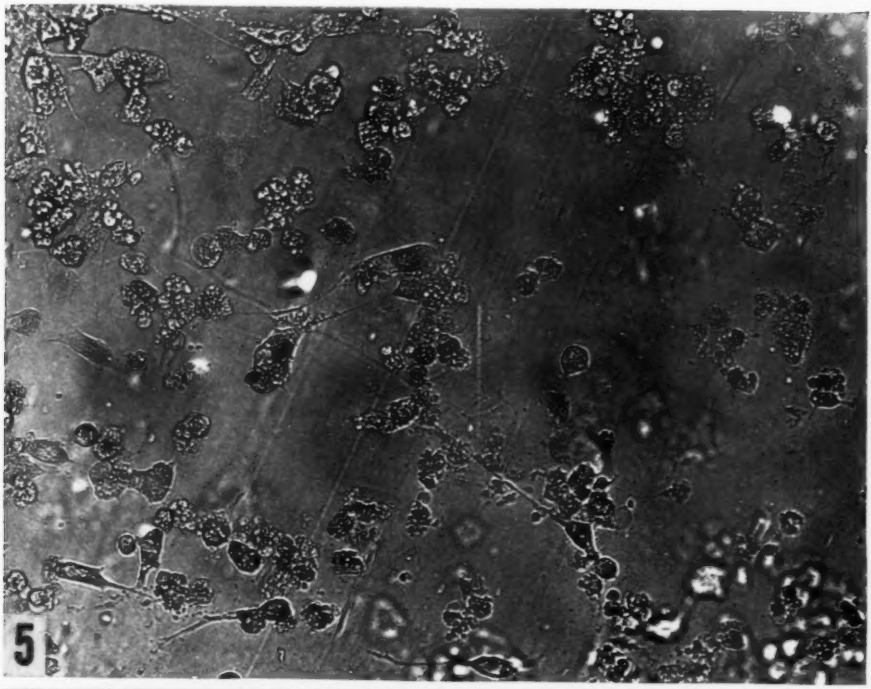

4

PLATE 80

FIGURE 5.—An almost completely necrotic culture of strain L mouse cells grown under perforated cellophane. This culture was allowed to become necrotic by not renewing the culture fluid. × 200.

FIGURE 6.—Same culture as that of figure 5 after 0.1 M citric acid was added. At the higher magnification used for counting the nucleus indicated by the arrow would be enumerated as a healthy nucleus, while nuclei such as that indicated by the crossed arrow would be counted as necrotic cell nuclei. × 200.


5

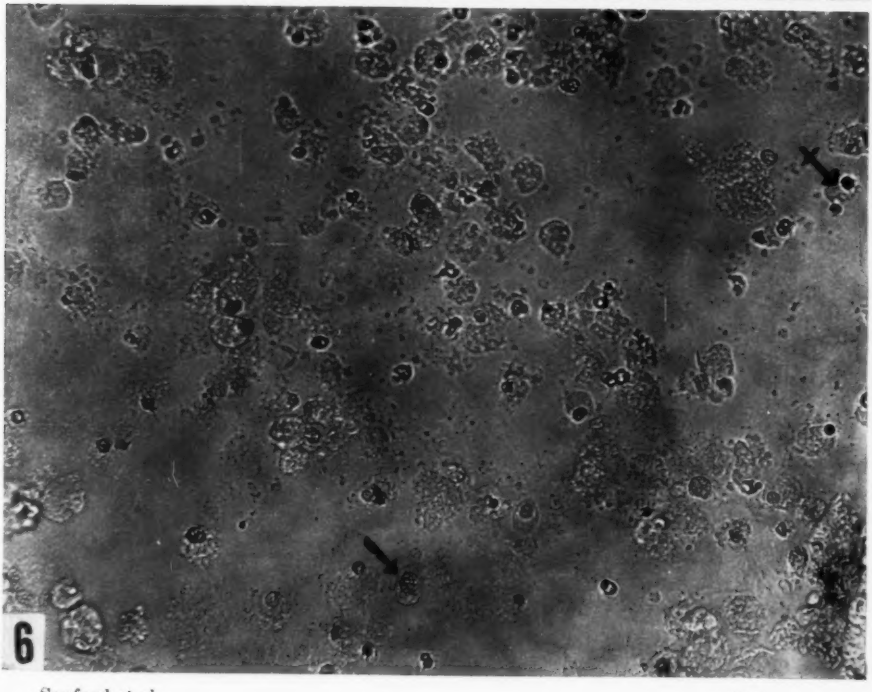
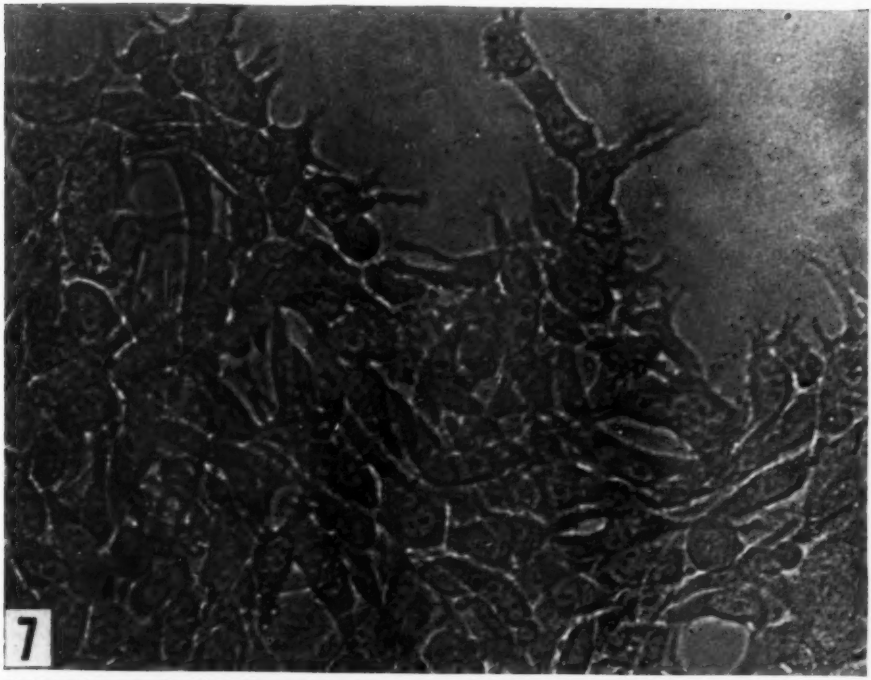

6

PLATE 81

FIGURE 7.—Strain L mouse cells growing in plasma clot in D–3.5 Carrel flask, photographed at the glass interface of the culture. × 400.

FIGURE 8.—Cells of the same field as figure 7 about 15 minutes after 0.1 M citric acid was added. × 400.


7

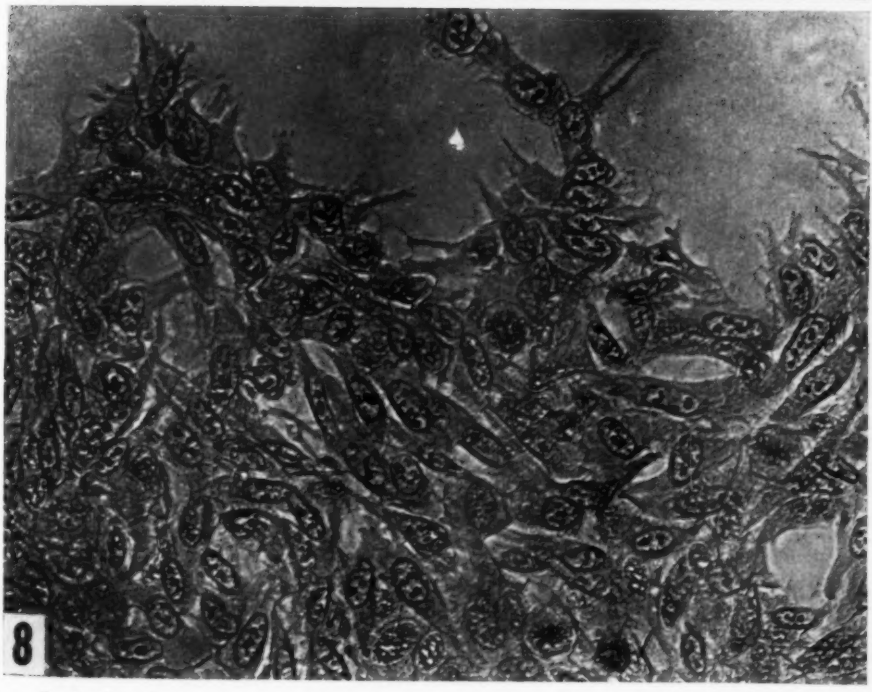
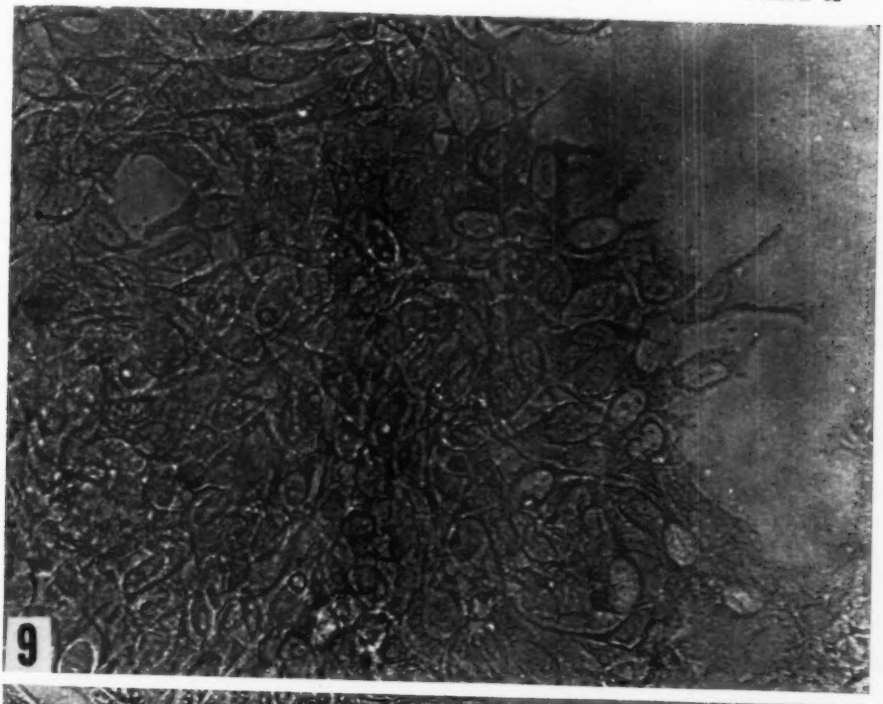
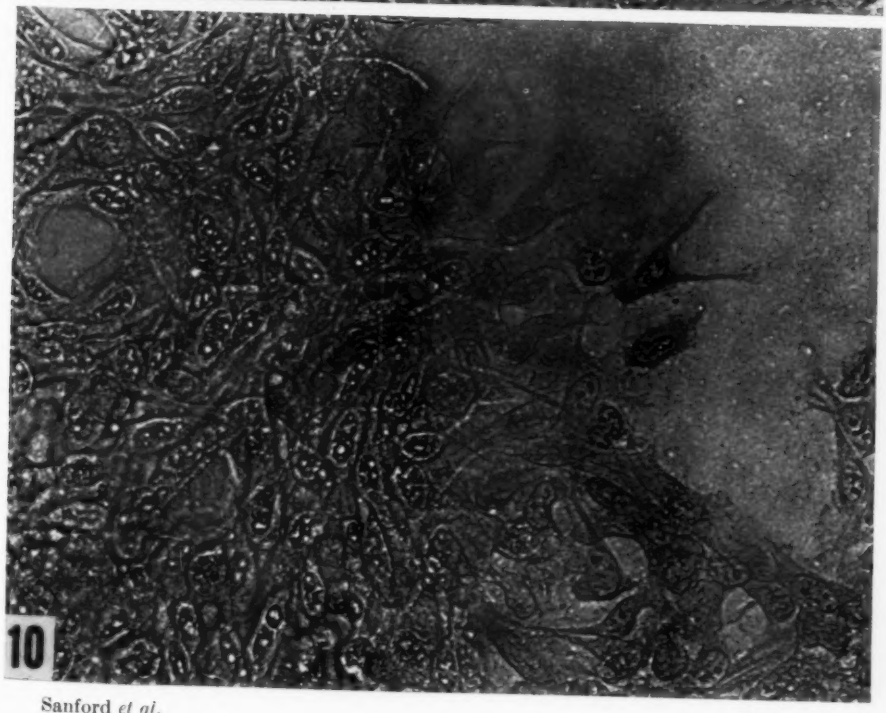

8

PLATE 82

FIGURE 9.—Cells of the culture of figure 7 after citric acid was withdrawn and the culture was washed with a nearly saturated solution of Na_2CO_3. × 400.

FIGURE 10.—Cells of the same field as figure 9 after initial treatment with citric acid, wash with solution of Na_2CO_3, and final wash with citric-acid solution. × 400.





Sanford *et al.*

929548—51——14

THE EXCRETION OF RADIOACTIVITY DURING A FOUR-DAY PERIOD FOLLOWING THE FEEDING OF CARBON 14-LABELED 2-ACETYLAMINOFLUORENE TO RATS [1] [2]

JOHN H. WEISBURGER, ELIZABETH K. WEISBURGER[3] and HAROLD P. MORRIS, *National Cancer Institute, National Institutes of Health, U. S. Public Health Service, Bethesda, Md.*

INTRODUCTION

The excretion of diazotizable material in the urine of rats fed 2-acetylaminofluorene (AAF) was found by Morris and Westfall (*7*) to be related to the individual dose and the interval between doses. This was established by the use of a colorimetric chemical method based on the presence of an aromatic amino group. A maximum of 45 percent of the dose was recovered after an intake of 16.0 mg. per 100 gm. of body weight per week administered in 3 equal stomach-tube instillations. Negligible amounts were found after the ingestion of 0.25 mg. per 100 gm. of body weight per week. This suggested that the rat could metabolize a certain quantity of AAF with destruction or masking of the aromatic amino group, while larger doses would spill over into the urine as metabolites still containing the diazotizable group. No diazotizable material was found in the feces, irrespective of the dose fed. In the present investigation tracer techniques, as used in a study of the distribution of AAF (*6*), were applied to the problem of the excretion of the carcinogen in order to gain a more complete picture of the disposal of ingested AAF by rats.

METHOD

Young female Buffalo rats, weighing 120 gm. were given by stomach tube a solution of either AAF–9–C^{14} or AAF–ω–C^{14} in propylene glycol at the level of 10 mg. per 100 gm. of body weight and placed in a metabolism chamber. This was built from a 3-liter round-bottom flask provided with air inlets and outlets, and tubes for the separate collection of feces and urine.[4] The top of the cage was made air-tight by being

[1] Received for publication October 17, 1950.

[2] The radiocarbon used in this investigation was obtained on allocation from the United States Atomic Energy Commission after being synthesized into 2-acetylaminofluorene by F. E. Ray and C. R. Geiser (*8*).

[3] Research Fellow, National Cancer Institute, U. S. Public Health Service.

[4] Dr. S. Wollman, National Cancer Institute, is the original designer of these chambers.

closed with two sheets of dental rubber, between which was inserted a metal disk of slightly larger diameter than the top opening of the cage. The animals had free access to food and water.

Carbon-dioxide-free air was drawn through the system at the rate of 300 ml. per minute. The exhaled carbon dioxide was collected in a solution of sodium hydroxide. This was made by diluting a 50-percent solution of sodium hydroxide (2 ml. for each hour of the collecting period) to 80 ml. (volume of absorbing tower) with carbonate-free water. Fractions were collected at the intervals indicated in table 1. For analysis, the solution contained in the tower was made up to an exact volume, and barium carbonate precipitated from an aliquot, after the addition of ammonium chloride in an amount equivalent to the sodium hydroxide originally present. The carbonate was plated and counted.

The urine, in weighing bottles, was taken to dryness in a vacuum desiccator in order to obtain the specific activity of the dry residue. After being weighed, the residue was dissolved in water and made up to 5 ml. To a 1-ml. aliquot in an ice bath, 1 ml. of fuming sulfuric acid (20 percent SO_3) was added carefully, and the two layers were mixed slowly. The resulting solution was submitted to the wet-combustion method of Van Slyke, as described previously (*6*).

At the end of 88 or 96 hours in the metabolism cage, the animal was killed by heart puncture, and the more important organs and tissues were dissected. The feces and tissues were analyzed for radioactivity by the procedure given in the preceding paper (*6*) of this series.

RESULTS

The rapid removal of the acetyl group of AAF, already reported by Morris, Weisburger, and Weisburger (*6*), was confirmed by the high radioactivity of the respired carbon dioxide from rat No. 6 receiving AAF-ω-C^{14} (table 1). The specific activity of the barium carbonate (counts per minute per milligram of barium carbonate) was fairly high at the beginning of the experiment and reached a maximum between the 2d and 4th hours. It then declined somewhat to the 30th hour, when the drop in activity was more rapid. After the 48th hour relatively small amounts of activity were observed. In 88 hours a total of 40.6 percent of the recovered activity had been excreted in the breath.

The maximum specific activity of the urine occurred between the 23d and 30th hour, but high activities were found from 0 to 48 hours. After that a rather sudden decline was observed. The urine accounted for 36.5 percent of the total recovered activity. The feces contained 11.8 percent of the total recovered activity. The highest specific activity of the feces was reached in the 30- to 36-hour period, with high levels between 12–48 hours and a rapid decline in specific activity after 48 hours.

At the end of 88 hours, 11.1 percent of the recovered activity remained in the animal (table 2), the carcass accounting for 7.3 percent and the gastrointestinal tract for 1.6 percent. The specific activity of fatty tissue was on the average somewhat higher than that of muscular tissue. The

TABLE 1—*Excretion of radioactivity in the rat after oral administration of a propylene glycol solution of C^{14}-labeled 2-acetylaminofluorene*

Rat No. 6, wt. 120 gm., 1-ml. solution, 12.93 mg. AAF–ω–C^{14}

Hours	Respired CO_2			Urine			Feces		
	Total c./min.	Spec. activity	% of total	Total c./min.	Spec. activity	% of total	Total c./min.	Spec. activity	% of total
0–2	22, 100	9. 8	2. 4						
2–4	27, 700	12. 8	2. 9						
4–6	20, 700	9. 3	2. 2	27, 800	163. 6.	3. 0			
6–12	47, 800	4. 8	5. 1	27, 000	266. 0	2. 9			
12–23	109, 000	6. 0	11. 6	50, 000	378. 7	5. 3	13, 800	178. 7	1. 5
23–30	75, 200	6. 1	8. 0	133, 000	899. 2	14. 1			
30–36	28, 400	2. 4	3. 0	74, 000	478. 8	7. 9	51, 900	194. 6	5. 5
36–48	21, 500	1. 2	2. 3	24, 300	122. 7	2. 6	16, 500	120. 1	1. 8
48–54	4, 300	. 5	. 5	1, 830	33. 0	. 2			
54–60	4, 130	. 4	. 4	1, 630	13. 8	. 2			
60–72	11, 700	. 5	1. 2	1, 840	10. 2	. 2	20, 600	61. 0	2. 2
72–78	3, 130	. 4	. 3						
78–84	3, 540	. 4	. 4	810	6. 3	. 1	5, 570	23. 0	. 6
84–88	1, 840	. 4	. 2	485	4. 9	. 05	2, 240	19. 0	. 2
	381, 000		40. 6	343, 000		36. 5	111, 000		11. 8
Rat No. 7, wt. 124 gm., 1-ml. solution, 12.07 mg. AAF–9–C^{14}									
0–4	600	0. 1	0. 04	38, 800	568	2. 6			
4–10	790	. 09	. 05	136, 000	810	9. 0			
10–22	1, 500	. 08	. 1	354, 000	1, 110	23. 5	127, 000	560	8. 4
22–34	680	. 06	. 05	368, 000	1, 190	24. 4	189, 000	654	12. 6
34–46	1, 700	. 08	. 1	36, 500	167	2. 4	27, 200	502	1. 8
46–58	1, 800	. 1	. 1	13, 000	65	. 9	45, 800	237	3. 0
58–70	1, 300	. 07	. 08	6, 250	30	. 4	19, 000	146	1. 3
70–82	1, 000	. 05	. 06	4, 590	24	. 3	10, 000	77	. 7
82–96	2, 200	. 08	. 2	6, 620	20	. 4	13, 700	42	. 9
	12, 000		0. 8	964, 000		63. 8	432, 000		28. 6

Harderian glands had the highest specific activity of all tissues or organs, being four times larger than the next highest (cecum and contents) and approximately ten times larger than the average specific activity of most organs.

The inability of the rat to oxidize significant amounts of the 9-carbon atom of the fluorene nucleus to carbon dioxide was demonstrated by the negligible amount of radioactivity in the breath of rat No. 7 fed AAF–9–C^{14}. The activity found over a period of 96 hours was close to the limit of experimental error and the values shown (table 1) are not significant.

The major path of excretion, on the other hand, seemed to be via the urine, which in 96 hours accounted for 63.8 percent of the total recovered activity. A high level was encountered in the urine from the beginning of the experiment, a maximum being observed between the 10th and the 34th hour. In that period 48 percent of the total activity was recovered. Thereafter a sudden decrease took place, and relatively low activities were excreted between 34 and 96 hours.

A considerable portion of the activity, 28.6 percent, was eliminated in the feces in 96 hours. The peak specific activity in the feces occurred in the period between 10 and 46 hours, with a maximum at 22–24 hours.

TABLE 2.—*Distribution of radioactivity in the rat after oral administration of a propylene glycol solution of C^{14}-labeled 2-acetylaminofluorene*

	Rat No. 6, wt. 120 gm.; 88 hr., 1 ml. solution; 12.93 mg. AAF-ω-C^{14}			Rat No. 7, wt. 124 gm.; 96 hr., 1 ml. solution; 12.07 mg. AAF-9-C^{14}		
	Total c./min.	Spec. activity	Percent total	Total c./min.	Spec. activity	Percent total
Stomach [1]	2, 130	4. 4	0. 2	1, 220	5. 2	0. 08
Small intestine [1]	9, 820	7. 0	1. 0	5, 650	4. 3	. 4
Cecum [1]	3, 970	10. 6	. 4	8, 290	22. 6	. 6
Large intestine [1]	360	2. 1	. 04	230	1. 7	. 01
Respiratory CO_2	381, 000		40. 6	12, 000		. 8
Urine	343, 000		36. 5	964, 000		63. 8
Feces	111, 000		11. 8	432, 000		28. 6
Liver	4, 770	3. 3	. 5	7, 270	4. 4	. 5
Kidneys	1, 550	5. 1	. 2	1, 170	4. 4	. 08
Lungs	1, 470	6. 3	. 2	610	3. 6	. 04
Bladder	80	5. 2	. 009	70	4. 4	. 005
Heart	480	3. 6	. 05	262	2. 2	. 02
Blood	96	2. 0	. 1	3, 480	4. 9	. 2
Spleen	300	4. 7	. 03	200	2. 9	. 01
Brain	1, 860	6. 1	. 2	390	1. 3	. 03
Bones (defatted)	4, 690	. 8	. 5	3, 030	. 7	. 2
Bone fat	1, 060	1. 6	. 1	1, 440	1. 8	. 1
Harderian glands	2, 430	43. 3	. 3	162	3. 3	. 01
Salivary glands	690	4. 1	. 07	60	. 3	. 003
Carcass	68, 400	2. 2	7. 3	64, 200	1. 8	4. 3
Total recovered counts	939, 000		100	1, 510, 000		100
Total administered counts	918, 000			1, 410, 000		
Recovery	102%			107%		

[1] And contents.

Moderate amounts were found up to the 70th hour, and relatively small quantities after that time.

After 96 hours this animal retained 9.6 percent of the recovered activity (table 2), of which 4.3 percent was in the carcass and roughly 1.3 percent in the gastrointestinal tract. The blood, bladder, kidneys, and liver had the highest specific activity, and the Harderian glands a fairly high activity.

DISCUSSION

Since more than 40 percent of the ingested activity of AAF–ω–C^{14} appears in the breath as $C^{14}O_2$, it must be inferred that deacetylation of AAF occurs to at least that extent. Moreover, some of the activity in the urine, feces, and organs may be attributed to compounds resulting from the metabolism of acetate, separated from the fluorene portion of AAF. The present study permits no quantitative conclusions as to the extent of deacetylation *in vivo*, except that the minimum is approximately 40 percent. In view of this finding, it is not clear why AAF in many experiments (*5*, *10*) appears to be more carcinogenic than 2-aminofluorene (AF) and causes more widespread tumors. Possibly the deacetylation is reversible, so that the radioactivity observed in the breath and tissues results from an exchange of the labeled acetate from the side chain and the acetate from the body pool.

In rat No. 6, lipoid-rich tissue, such as the brain, had a higher activity than in rat No. 7. This finding suggests that after removal of the acetyl group of AAF–ω–C^{14}, part of the labeled acetate may be incorporated into the body fat.

The fact that over a period of 4 days insignificant amounts of activity appeared in the breath of a rat fed the 9-labeled compound shows that neither AAF itself, nor any of the possible breakdown products, are oxidized *in vivo* with liberation of the 9-carbon atom as carbon dioxide. This inability of the animal to convert fluorene to carbon dioxide parallels the findings from tracer studies with 1,2,5,6-dibenzanthracene-9,10–C^{14} (*4*), Benadryl–α–C^{14} (*2*, *3*), Methadone–2–C^{14} (*1*), and Pentobarbital–2–C^{14} (*9*).

Only 36.5 percent of the activity given as AAF–ω–C^{14} was excreted in the urine, while the urine was the major path of excretion of the 9-labeled compound, with 63.8 percent eliminated in this manner in 4 days. The excretion of activity in the urine began immediately after the administration of either labeled compound, and reached the highest levels up to the 34th hour for AAF–9–C^{14} and the 48th hour for AAF–ω–C^{14}. Thereafter, a sharp, pronounced decrease in urinary activity took place. At those times approximately 81.7 and 82 percent, respectively, of the total activity had been excreted. This may be compared to a maximum of 45 percent of the dose recovered in the urine by Morris and Westfall (*7*) over a period of 1 week. Moreover, these authors found no diazotizable materials in the feces after ingestion of AAF, while in this research 28.6 percent of the activity after feeding the 9–labeled compound was

accounted for in the feces. The compound or compounds carrying the activity, therefore, could not have been aromatic amines. This indicates that AAF undergoes a considerable transformation in the course of its metabolism in the rat. Experiments now in progress using rats with biliary fistulas, will perhaps throw some light on the possible loci of these reactions.

A concentration of AAF or metabolites in certain target organs is still apparent 96 hours after the feeding of a single dose of AAF–9–C^{14}. Disregarding the gastrointestinal tract, the specific activity of the following organs was significantly higher than the carcass level: liver, kidneys, lungs, bladder, blood, spleen, and Harderian glands. The activity of most organs, as expected, was lower than previously reported (*6*) for the distribution 6 hours after feeding the labeled compounds, indicating a progressive elimination of the metabolites over a period of several days.

SUMMARY

The excretion of radioactivity by rats over a period of approximately 4 days after a single dose of ω–C^{14} or 9–C^{14} labeled 2-acetylaminofluorene (AAF) is reported.

The breath contained insignificant amounts of radioactivity after feeding AAF–9–C^{14}, while more than 40 percent was found after feeding AAF–ω–C^{14}. The long-term experiments thus confirm the occurrence of the deacetylation of AAF *in vivo* found in short-term experiments.

The urine accounted for 63.8 and 36.5 percent of the activity from the ring-labeled and the side-chain-labeled compounds, respectively. Thus, the urine was the major path of excretion of the activity from AAF–9–C^{14}.

The feces contained 28.6 percent of the recovered radioactivity from the AAF–9–C^{14}. This is in striking contrast to the failure to detect AAF in the feces by tracer studies of 6 hours or less in duration, or by chemical methods depending on the presence of a diazotizable amino group. This is the first evidence that the feces contain important metabolites no longer diazotizable.

REFERENCES

(*1*) Elliott, H. W., Chang, F. N-H., Abdou, I. A., and Anderson, H. H.: The distribution of radioactivity in rats after administration of C^{14}-labeled Methadone. J. Pharmacol. & Exper. Therap. 95: 494–501 (1949).

(*2*) Glazko, A. J., McGinty, D. A., Dill, W. A., Wilson, M. L., and Ward, C. S.: Biochemical studies on diphenhydramine (Benadryl). III. Application of radioactive carbon to metabolic studies of Benadryl. J. Biol. Chem. 179: 409–416 (1949).

(*3*) Glazko, A. J.: Private communication.

(*4*) Heidelberger, C., and Jones, H. B.: The distribution of radioactivity in the mouse following administration of dibenzanthracene labeled in the 9 and 10 positions with Carbon 14. Cancer 1: 252–260 (1948).

(*5*) Morris, H. P., Dubnik, C. S., and Johnson, J. M.: Studies of carcinogenic action of 2-nitro-, 2-amino-, 2-acetylamino-, and 2-diacetylaminofluorene after ingestion and after painting. J. Nat. Cancer Inst. 10: 1201–1213 (1950).

(*6*) MORRIS, H. P., WEISBURGER, J. H., and WEISBURGER, E. K.: The distribution of radioactivity following the feeding of carbon 14-labeled 2-acetylaminofluorene to rats. Cancer Research 10: 620–624 (1950).

(*7*) MORRIS, H. P., and WESTFALL, B. B.: Some studies of the excretion of diazotizable material after feeding 2-acetylaminofluorene to rats. Cancer Research 10: 506–509 (1950).

(*8*) RAY, F. E., and GEISER, C. R.: Synthesis of 2-acetylaminofluorene-9-C^{14} and 2-acetylaminofluorene-ω-C^{14}. Cancer Research 10: 616–619 (1950).

(*9*) ROTH, L. J., LEIFER, E., HOGNESS, J. R., and LANGHAM, W. H.: The metabolism of radioactive Pentobarbital in mice. J. Biol. Chem. 178: 963–966 (1949).

(*10*) WILSON, R. H., DEEDS, F., and COX, A. J.: The carcinogenic activity of 2-acetaminofluorene. IV. Action of related compounds. Cancer Research 7: 453–458 (1947).

THE EFFECT OF INGESTION OF THIOURACIL ON STRAIN C MICE [1]

HAROLD P. MORRIS, CELIA DUBNIK GREEN, and ALBERT J. DALTON, *National Cancer Institute, National Institutes of Health, U. S. Public Health Service, Bethesda, Md.*

The use of an antithyroid compound, thiourea or thiouracil, to specifically block the secretions of the thyroid gland has been shown to produce and maintain a hormonal imbalance for prolonged periods (*1–4*). When such a procedure was initiated in virgin strain C3H female mice at an early age there resulted a drastic arrest in mammary-gland development, a reduction in the number of hyperplastic nodules, and a pronounced lowering of the incidence of spontaneous mammary tumors. Estrous cycles were irregular or infrequent (*2*). The hyperplasia and hypertrophy of the thyroid gland were accompanied by adrenal and pituitary changes, and atrophy of the ovaries and uterus. Thyroid metastases to the lungs of treated mice were frequently observed (*3*).

It was recognized that other strains of mice might differ in their susceptibility to the creation or maintenance of a hormonal imbalance produced by antithyroid compounds. The object of this experiment, therefore, was to compare the response of another inbred strain of mice to the prolonged ingestion of thiouracil, in order to determine whether or not the effects on thyroid neoplasia (*5*), mammary-gland development, and spontaneous mammary-tumor formation observed in the C3H female mouse could be duplicated. The strain C mouse was selected because presumably the virgin C female has two of the three factors necessary for the development of spontaneous mammary tumors; *i. e.*, hereditary susceptibility and a favorable hormonal balance. The milk agent could be introduced by foster-nursing by C3H dams.

MATERIALS AND METHODS

Virgin strain C (B alb C) mice obtained from the N. C. I. colony after having been foster nursed by strain C3H mothers were divided at 10 weeks of age into 2 groups comprising 62 control and 65 thiouracil-fed mice.[2] The diet, similar to the one used previously (*2*), was composed

[1] Received for publication October 23, 1950.

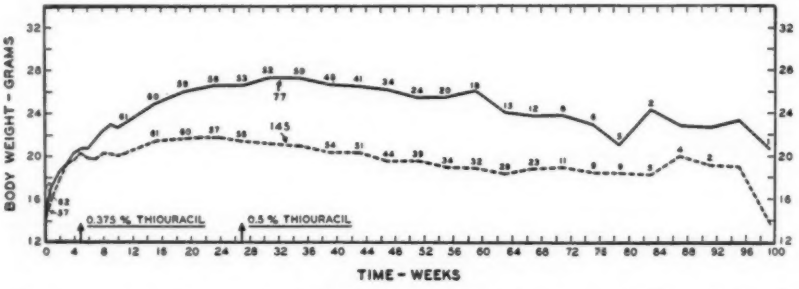
[2] Seventeen additional foster-nursed females were fed the thiouracil diet, and sacrificed for histologic examination at various intervals during the early part of the experiment. A final group of non-foster-nursed virgin females was divided into 2 groups of 10 controls and 12 thiouracil-fed animals. Non-foster-nursed animals were included to study the effect of treatment in the absence of the milk agent.

of natural foodstuffs. This diet, containing at the start 0.37-percent thiouracil,[3] was fed to the experimental mice for 26 weeks and then the amount of drug was raised to 0.5 percent. Vaginal smears were taken daily, except Sunday. Whole mammary-gland mounts were prepared according to the technique of Morris, Dubnik, and Dalton (*1*). All animals were weighed weekly throughout the experiment. Tumor-bearing mice were killed when their mammary tumors attained a diameter of 0.5–1.0 cm. Non-tumor-bearing animals were sacrificed when death appeared imminent.

EXPERIMENTAL RESULTS

Body Weight

Two groups of virgin C female mice, comprising 57 [4] and 62 animals, increased in average body weight at the same rate, until after the addition of thiouracil to the diet of the former group (text-fig. 1). Growth as indicated by the body weight of the treated animals slowed down considerably after thiouracil ingestion was begun, with an average body-weight increase of approximately 1.5 grams during the first 26 weeks of the experiment. A slight drop in average weight resulted after that time, when the thiouracil level was raised, and there was approximately a 6-gram difference in average weight between the 2 groups beyond about the 26th week of treatment.



TEXT FIGURE 1.—Average body weights of control No. 77 and thiouracil-treated No. 145 mice.

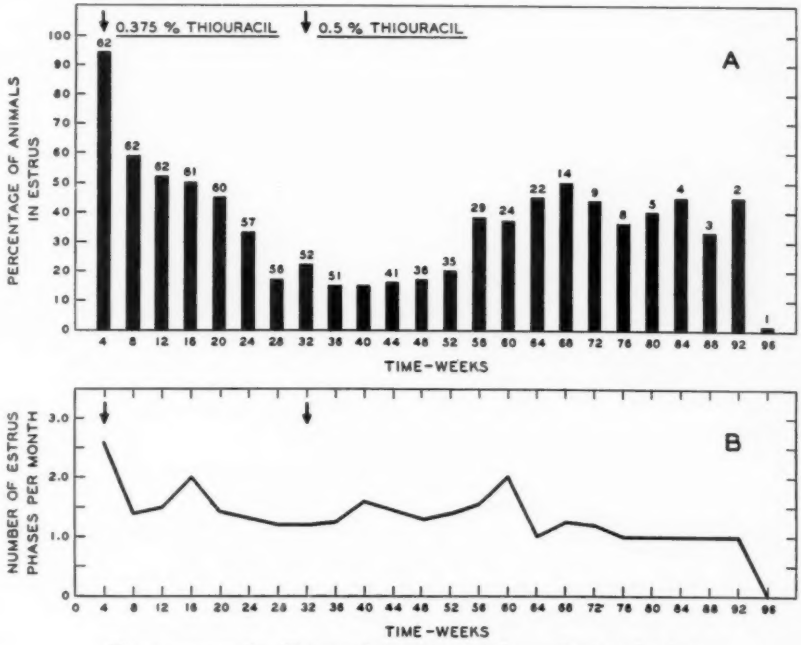
Estrous Cycles

No vaginal smears were obtained in the control animals, but smears were taken of the treated group during the period of thiouracil ingestion. Of the 62 animals examined during the first month of thiouracil ingestion 95 percent were in estrus at least once [text-fig. 2A (three animals that died early in the experiment were excluded)]. Thereafter, the percentage of animals undergoing estrous cycles dropped continuously, and from the 7th through the 13th month approximately 17 percent of the group were

[3] Thiouracil was generously supplied by the Lederle Laboratories, Pearl River, N. Y., through the courtesy of Dr. S. M. Hardy.

[4] Eight treated mice dying between 3 and 12 weeks of thiouracil ingestion were excluded from the average.

cycling. About 50 percent of the animals were still undergoing sporadic estrous cycles after the 13th month, but the same animals were not in estrus each month. The average number of estruses per month varied from a maximum of 2.6 to a minimum of about 1.3, with a slight rise occurring after the 12th month (text-fig. 2B).


0.375 % THIOURACIL
0.5 % THIOURACIL
A
PERCENTAGE OF ANIMALS IN ESTRUS
62 62 62 61 60 57 56 52 51 41 38 35 29 24 22 14 9 8 5 4 3 2 1
TIME-WEEKS
B
NUMBER OF ESTRUS PHASES PER MONTH
TIME-WEEKS

TEXT FIGURE 2.—Estrous behavior of thiouracil-treated mice.

A. Percentage of animals manifesting estrus each month. (The number above each bar represents the number of animals alive.)

B. Average number of estrous phases per month.

MAMMARY-GLAND DEVELOPMENT

The mammary-gland structure of both control and treated mice showed considerable variation, which was further complicated by age and foster nursing, and in treated animals by the additional effects of thiouracil ingestion. In order to note and describe these changes, the duct diameters, the degree of lateral budding, the complexity of branching, and the extent of involution or under-development, as the case might be, were graded numerically as follows:

A. Duct diameter: 1=majority of ducts, thin or hairlike in character (figs. 5 and 8); 2=normal distribution of narrow to wide ducts (fig. 2); 3=dilatation, side branches wider than duct or origin (figs. 1 and 4).

B. Lateral budding: 0=none (fig. 4); 1=scattered (fig. 6); 2=moderate (fig. 3); 3= abundant, (figs. 1 and 2).

C. Branching: 1=simple (fig. 5); 2=intermediate (fig. 4); 3=complex (fig. 2).

D. Atrophy or underdevelopment: 0=none (fig. 2); 1=involving peripheral ⅓ of gland (fig. 6); 2=involving peripheral ⅔ of gland (fig. 7); 3=both basal and peripheral regions involved (fig. 4).

Control Animals

The great amount of individual variation in the gross anatomy of the mammary glands of the 42 control animals examined, ranging in age from 131 to 612 days, did not entirely obscure some patterns of progressive change. There was a tendency for side ducts to be wider than their ducts of origin, (figs. 1 and 4, *cf.* fig. 2). Ducts 3 to 4 times the diameter of comparable ducts in younger animals were frequently present, especially in the basal region of the gland. The glands of all animals examined after 200 days of age showed this tendency in varying degree.

The amount of lateral budding and complexity of branching increased with advancing age. The peak of such development was reached during the 300- to 400-day-age interval, when budding was most profuse and branching most complex. There was a tendency toward varying amounts of involution after 400 days, (fig. 4). The earliest evidence of atrophy was seen in a 250-day-old animal and consisted of loss of budding and peripheral side branches ending in blunt or rounded projections. Involution was consistently present in animals sacrificed beyond 425 days of age. There was also a tendency for some peripheral ducts in the older animals to become somewhat narrower, but the dilated ducts were still present in the oldest animals.

Hyperplastic nodules, or areas of alveolar hyperplasia, were present in the mammary glands of 25 of the 42 foster-nursed control animals. The earliest nodules observed were in an animal killed at 188 days of age. Six of the 25 animals with mammary tumors did not appear to have any hyperplastic nodules, while nodules were present in 6 animals without tumors. There were 81 nodules in 24 mice, or an average of 3.7 nodules per animal, while the 1 other nodule-bearing animal had 51 areas of alveolar hyperplasia.

The mammary glands of 12 non-foster-nursed control mice of the same strain and age distribution as the foster-nursed controls were also examined. This group presented roughly the same mammary picture; *i. e.*, ductal dilatation in some glands, although budding did not appear to be as extensive as in the foster-nursed animals. The essential difference was the absence of hyperplastic nodules.

Thiouracil-treated Animals

The mammary glands were examined from 58 thiouracil-treated foster-nursed animals ranging from 124 to 822 days of age. These glands presented not only considerable variation from animal to animal of the same age but also variations in different glands from the same animal (figs. 3 and 5). The ducts of the glands from treated mice, in contrast to

the control glands, were considerably reduced in diameter, and the pronounced tendency toward localized dilatation of individual ducts was not very evident. Scattered ductal dilatation was sometimes present but on a greatly reduced scale (fig. 7). The lateral ducts were usually quite thin and sometimes almost hairlike in appearance (figs. 5–7). Lateral budding was sparse even in young animals (fig. 8), and the increase in acinar structures, which occurred in control animals, was observed in the treated animals but to a lesser extent about 150 days later, when the animals were between 450 and 550 days of age (figs. 3 and 6). Ductal branching was reduced in the treated animals, and the glandular tree never reached the complexity or extent attained by the control animals.

Hyperplastic nodules appeared in only two animals, a total of six was present.

The entire mammary pattern of non-foster-nursed, treated mice was much simplified, and little or no acinar structures were present. There was a slight tendency for ducts to become even more slender with increasing intervals of treatment, and no ductal dilatation was present. Hyperplastic nodules were not observed in glands of non-foster-nursed, treated mice.

The arbitrarily assigned numerical values used in grading the levels of structural development were summarized by 100-day intervals. No values beyond 600 days were used because too few animals remained. An estimation of mammary development by use of such a scheme was thus obtained. The average values indicate that the control levels for branching, ductal diameters, and budding were almost always higher than those for the treated mice, and the involution levels were reversed, being higher for the treated than for the control mice.

Survival and Tumor Incidence

The survival of the 2 groups was similar, with an average of 373 days for the 62 control and 398 days for the 65 thiouracil-treated animals.

Mammary tumors appeared only in the control group. Forty-eight percent of 60 control animals, 20 weeks of age or older, developed tumors. The first tumor appeared in an animal 161 days old and the last in a mouse 515 days old, with an average age at tumor appearance of 362 days.

DISCUSSION

Strain C mice after ingesting thiouracil for about 12 weeks ceased increasing in body weight; thereafter, they were on the average about 20 percent lighter than their controls. In the thiouracil-treated C and C3H mice this average retardation in weight took place to about the same extent and at about the same time (*2*). The same concentration of thiouracil, during the same age period, was given to both C and C3H mice.

The effect of prolonged thiouracil ingestion on estrous behavior in strain C mice was somewhat different from that produced in strain C3H. Complete suppression of estrus occurred in all C3H mice after 4 weeks of treatment and persisted for about 6 to 8 weeks. Estrous inhibition

in strain C animals was not so drastic, and it was only after 20 weeks of treatment that 75 percent of the animals showed temporary cessation or irregularity of estrous cycles.

Since the mammary-gland patterns of strains C and C3H mice are different, specific comparison of structural changes are difficult. The trends, however, are similar. The glands of treated mice of both strains possessed a much simpler mammary structure than their respective controls, and in both strains only about 3 or 4 animals had glandular development comparable to a control animal of similar age. Hyperplastic nodules or areas of alveolar hyperplasia were considerably reduced in both strains. The reduction in the number of nodules seemed to be more complete in the strain C gland, but perhaps the inhibition appeared more evident because the potential sites for their development were less numerous.

The greatest increase of mammary development and the most complex glands were found in the control strain C mice in the 300- to 400-day interval. The greatest arborization of the glands in the thiouracil-treated animals, however, was not noted until 150 days later, between 450–550 days. The few hyperplastic nodules that appeared in treated mice were found only during this later age interval. Estrous activity also increased during this 450- to 550-day period. The interval of decreased estrous activity occurred during the age interval in which mammary glands showed the least development. Huseby and Bittner (*6*) reported an increase in lateral budding in normal strain Z mice during the 300- to 400-day age interval. This increased budding was not apparent in either the control or the thiouracil-treated strain C3H mice (*2*).

Mammary-tumor production was completely suppressed in the C animals ingesting thiouracil, in contrast to an incidence of 19 percent in treated strain C3H mice; the absence of tumors in treated C mice may be related to the lower tumor incidence in control C mice compared to the much higher incidence in control C3H mice.

The average survival time of the two strains was quite similar—398 days for treated C mice compared to 436 days for treated C3H animals. Control C mice lived, on the average, 25 days less than the treated animals, while in the experiment on the strain C3H mice the difference in survival time between control and treated animals was 37 days.

The effects of prolonged ingestion of thiouracil, allowing for minor differences attributable to strain individuality, were, in general, quite similar in both strains. Normal estrous cycles were disrupted. Development of the mammary glands showed widespread inhibition, and formation of hyperplastic nodules was almost completely suppressed. Thyroid glands of both strains increased in size, yet only one case of pulmonary metastasis of thyroid tissue was found in strain C mice compared with the 11 cases in strain C3H (*3*). Despite the similar effects in the 2 strains, the correlations observed in strain C3H between thyroid-gland enlargement, mammary-gland development, and body weight were not present in strain C. One explanation, perhaps, for the apparent

lack of correlation could be that the range in thyroid weights in strain C mice was much less than occurred in strain C3H.

The mammary glands of the treated C animals were also much simpler, so that differences between animals was less obvious and could not be as readily or as accurately classified into stages of progressive development. The difference in results between the two groups, however, may be considered one of degree. This would suggest that the same sequence of changes takes place in both strains, and that the same mechanism involving the production of a hormonal imbalance not necessarily attributable to poor nutrition (*2*) is operable.

SUMMARY

The effects of ingestion of thiouracil by foster-nursed virgin strain C (B alb C) mice at a dietary level of 0.375 and 0.5 percent from 10 weeks of age until death were studied.

The treated mice weighed on the average about 20 percent less than their foster-nursed controls.

Estrous cycles of animals ingesting thiouracil were partially suppressed, and their mammary glands failed to develop to any appreciable extent.

Hyperplastic nodules were absent in all but 2 of 62 treated animals. Twenty-five of the 62 control animals developed hyperplastic nodules.

No mammary tumors arose in 62 mice tbat ingested thiouracil, while 48 percent of 62 control animals developed tumors.

REFERENCES

(*1*) Morris, H. P., Dubnik, C. S., and Dalton, A. J.: Effect of prolonged ingestion of thiourea on mammary glands and appearance of mammary tumors in adult C3H mice. J. Nat. Cancer Inst. 7: 159–169 (1946).

(*2*) Dubnik, C. S., Morris, H. P., and Dalton, A. J.: Inhibition of mammary-gland development and mammary-tumor formation in female C3H mice following ingestion of thiouracil. J. Nat. Cancer Inst. 10: 815–841 (1950).

(*3*) Dalton, A. J., Morris, H. P., and Dubnik, C. S.: Morphologic changes in organs of female C3H mice after long term ingestion of thiourea and thiouracil. J. Nat. Cancer Inst. 9: 201–203 (1948).

(*4*) Money, W. L., and Rawson, R. W.: The experimental production of thyroid tumors in the rat exposed to prolonged treatment with thiouracil. Cancer 3: 321–335 (1950).

(*5*) Dalton, A. J., Morris, H. P., Striebich, M. J., and Dubnik, C. S.: Histologic changes in strain C mice following long term ingestion of thiouracil. J. Nat. Cancer Inst. 11:391–413 (1950).

(*6*) Huseby, R. A., and Bittner, J. J.: A comparative morphologic study of the mammary glands with reference to known factors influencing development of mammary carcinoma in mice. Cancer Research 6: 240–255 (1946).

PLATE 83*

FIGURE 1.—Control animal, #3370, 350 days old. Mammary development as extensive as in figure 2 but with greatly dilated ducts and numerous hyperplastic nodules.

FIGURE 2.—Control animal, #3412, 188 days old. Maximum amount of lateral budding and ductal branching, no dilatation of ducts.

FIGURE 3.—Thiouracil-treated animal, #3335, 511 days old. Profuse lateral budding, intermediate type branching, beginning dilatation of ducts.

FIGURE 4.—Control animal, #3392, 360 days old. Complete absence of lateral budding. Ducts thickened, one small hyperplastic nodule present.

*Whole mounts of mouse mammary glands. × 9.

929548—51——16

PLATE 84*

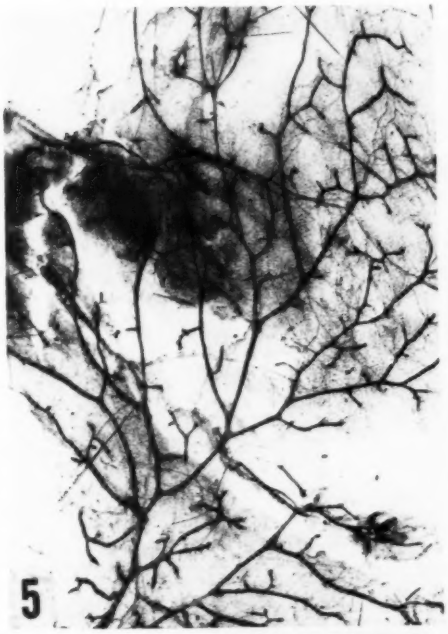
FIGURE 5.—Thiouracil-treated animal, #3321, 458 days old. Scattered end buds, ducts generally thin and hairlike with a slight suggestion of scattered ductal dilatation, simple branching of ducts.

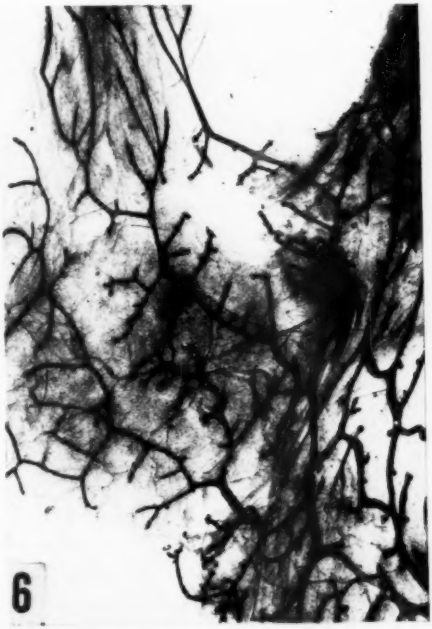
FIGURE 6.—Thiouracil-treated animal, #3345, 590 days old. Lateral budding present in basal half of gland; in peripheral half of gland branching simple with almost no alveolar budding.

FIGURE 7.—Thiouracil-treated animal, #3346, 694 days old. Scattered end buds, very simple branching, majority of ducts considerably reduced in diameter but some scattered ductal dilatation present.

FIGURE 8.—Thiouracil-treated animal, #3360, 161 days old. Note similarity to mammary glands shown in figures 5 and 7.

*Whole mounts of mouse mammary glands. × 9.


5


6

7

8

PRODUCTION OF MALIGNANCY *IN VITRO*. XII. BEHAVIOR OF RECOVERY CULTURES [1] [2]

EMMA SHELTON,[3] and WILTON R. EARLE, *National Cancer Institute, National Institutes of Health, U. S. Public Health Service, Bethesda, Md.*

INTRODUCTION

A preceding article in this series (*1*) describes the origin of 7 strains of fibroblasts, designated HW, D, H, J, L, N, and O, from the subcutaneous connective tissue of the strain C3H mouse and describes their subsequent behavior in culture. After exposure to the carcinogen, 20-methylcholanthrene, for varying periods of time, culture strains, H, J, L, N, and O gave rise to tumors when injected into strain C3H mice (*2*). Untreated control cultures of strains HW and D also produced tumors when injected into mice.

When these cell strains had been grown long enough in culture to permit analyses of their morphology and growth characteristics, and after they had been implanted into mice where they produced tumors that were carried by subinoculation for a period of years, the question arose, Will the cells from these tumor strains, when cultured, resemble the parent cultures that gave rise to the tumors? The initial purpose in culturing tissues from the 7 tumor strains reported here was to determine whether the tumor cells would preserve certain defined characteristics of the parent cultures after growing for a long period of time *in vivo*, or whether the physiology of the host would alter these characteristics.

In order to distinguish the cultures of the original cell strains from the cultures of the tumor strains, the latter will be termed "*recovery*" cultures.

MATERIALS AND METHODS

The 7 tumor strains studied were carried in strain C3H, Andervont substrain, female mice. The history of the individual tumors from which the recovery cultures originated is summarized in table 1, and is briefly described as follows:

Tumor strain HW arose from control (untreated) H cultures injected

[1] Received for publication October 30, 1950.

[2] This article represents a portion of a thesis presented by the senior author to the faculty of The George Washington University in partial completion of the requirements for the degree of Master of Arts.

[3] The senior author wishes to express grateful appreciation to Dr. Ira B. Hansen, Professor of Zoology, The George Washington University, for his understanding co-operation in the preparation and presentation of this material as a thesis.

929548—51——17

into mice on September 9, 1942. Tissue for the recovery cultures was explanted from a tumor that was 14 days old in the 52d generation of subinoculation in the mouse.

Tumor strain D arose from control (untreated) D cultures injected into mice on December 20, 1944. Tissue for the recovery cultures was explanted from a tumor that was 35 days old in the 3d generation of subinoculation in the mouse. This tumor was considerably younger than the others used for the recovery cultures.[4]

Tumor strain H arose from carcinogen treated (6 days) H cultures injected into mice on December 1, 1942. Tissue for the recovery cultures was explanted from a tumor that was 14 days old in the 50th generation of subinoculation in the mouse.

TABLE 1.—*Chart summarizing the experimental history of the cell strains used in this study*

Strain	Days in carcinogen	Days out of carcinogen before injection	Total *in vitro* life in days	Total *in vivo* life in days	Days *in vitro* "recovery" series
HW	Control	----------	691	931	180
D	Control	----------	1, 523	99	180
H	6	355	774	848	180
$J_{(1)}$	32	270	715	907	309
$J_{(2)}$	32	270	715	1, 137	79
L	111	221	745	877	251
N	184	239	715	907	112
O	406	56	754	868	180

Tumor strain J arose from carcinogen-treated (32 days) J cultures injected into mice on October 3, 1942. Tissue for the recovery cultures $J_{(1)}$ was explanted from a tumor that was 14 days old in the 51st generation of subinoculation in the mouse.

On November 14, 1945, a second series of explants $J_{(2)}$ was taken from J tumors. Tissue was explanted from 3 separate tumors that were 19 days old in the 64th generation of subinoculation in the mouse.

Tumor strain L arose from carcinogen-treated (111 days) L cultures, injected into mice on November 2, 1942. Tissue for the recovery cultures was explanted from a tumor that was 14 days old in the 52d generation of subinoculation in the mouse.

Tumor strain N arose from carcinogen-treated (184 days) strain N cultures injected into mice on October 3, 1942. Tissue for the recovery cultures was explanted from a tumor that was 14 days old in the 52d generation of subinoculation in the mouse.

Tumor strain O arose from carcinogen-treated (406 days) strain O cultures injected into mice on November 11, 1942. Tissue for the recovery cultures was explanted from a tumor that was 14 days old in the 52d generation of subinoculation in the mouse.

The carcinogen-treated cells had been exposed to one microgram of

[4] An older strain D tumor was originated on November 2, 1942, and was carried until the 24th generation with 100 percent "takes." After this time, increasing difficulty was experienced in transplanting the tumor, and it was eventually lost on December 21, 1944, in the 37th generation.

20-methylcholanthrene per milliliter of culture fluid for the periods of time indicated, but had been removed from the carcinogen and were then grown in normal culture fluid for at least 56 days before they were injected into mice.

Each tumor to be used for explantation was dissected from the mouse under aseptic conditions and placed in a sterile dish containing a few drops of balanced salt solution. A representative slice from each tumor was fixed at once in Zenker-formol solution for histologic examination. The tissues excised for culture and for animal inoculation were selected from areas of the tumor that were grossly free from necrosis. An attempt was made to cut for explantation approximately equal strips of tissue about 1 by 2 by 7 mm. in size from each of the tumors, but because of variations in consistency such uniformity was not always achieved. These strips were planted in Carrel D–3.5 flasks in clotted hen plasma mixed with horse serum, chick-embryo extract, and saline. All culture methods employed were similar to those described previously (*1*).

Tissue from each tumor was also injected by trocar into 10 strain C3H mice. The injections served to propagate the next generation of tumors, and also served as a check on the viability and tumor-producing ability of the tissue used for the cultures.

The tissues were allowed to grow in culture for 28 days, before reimplantation into new flasks. Each growth interval of 28 days was termed a culture generation. Measurements of the increase in the width of the cultures of each strain were made and averaged at the end of each generation. These average values were plotted against the time that the *original* cell strains had been exposed to the carcinogen. This followed the procedure previously used for measuring and plotting increase in width of the original cultures (*1*).

After the cells became well enough established to allow the planting of a fairly large number of cultures, some of them were again injected into strain C3H mice. A tabulation of these injections and the resulting tumors is given in table 2.

TABLE 2.—*The number and percentage of tumors produced by injection of* recovery-*culture strains into strain C3H mice*

SERIES 235

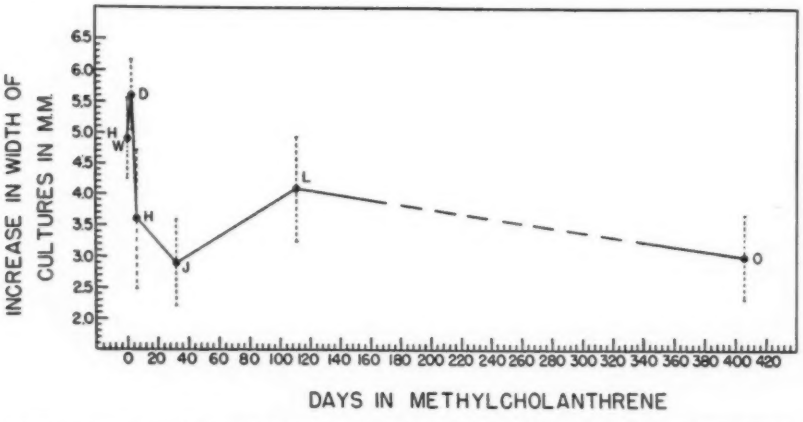
Strain	Number of injections	Number of tumors	Percentage
HW	12	12	100
D	23	23	100
H	16	13	81
$J_{(1)}$	63	0	0
$J_{(2)}$	34	1	3
L	24	17	71
N	4	2	50
O	10	8	80

Photomicrographs of the living cells in culture were made at intervals of 2, 10, and 28 days after explantation in the 1st generation. In the 2d,

3d, 4th, and 6th generations, photomicrographs were taken at 13, 13, 11, and 10 days after explantation, respectively. All photomicrographs were taken at a magnification of $\times 200$ by the method previously described (*3*). Photomicrographs of the stained tumor sections were taken at a magnification of $\times$ 400.

RESULTS

In order to compare the diameters of the recovery cultures with those of the original cultures, curves showing the average increase in width of the recovery cultures were plotted for each 28-day culture generation. The small number of cultures carried in the first 4 generations, and the necessary adjustments made by the cells to growth *in vitro*, made the curves for these generations of limited value. By the 6th generation *in vitro*, 12 to 15 cultures were carried for each strain, the cell morphology was stable, and the width of the cell clumps was more easily and accurately determined. The curve showing the average increase in width of the 6th generation of recovery cultures plotted against the number of days that the original cultures were exposed to the carcinogen is shown in text-figure 1.



TEXT-FIGURE 1.—Curve showing average increase in width of *recovery*-culture strains plotted against the time that the original cultures were exposed to 20-methylcholanthrene. Dotted lines through each point indicate standard deviations from the mean. Compare shape of curve with that of the original cultures shown in text-figure 2.

The tumor tissue injected into mice at the start of the experiment gave rise to 10 tumors out of 10 injections for each tumor strain except strain J. Tissue from the J tumor produced tumors in only 5 out of 10 mice injected. This tumor contained more than the usual amount of necrotic tissue and since the better part of the tumor was used for explantation, there was barely enough left for animal injection.

Histologic sections of each of the tumors that gave rise to the recovery cultures were examined with the hope of finding some correlation between

their morphology and the morphology of the cells in culture. For example, since many of the cultures of the strain J tumor consisted preponderantly of giant cells, it was felt that large cells might also be present in the tumor itself. But this was not the case. A careful examination of sections of all the tumors gave little indication of what one might expect to see in the recovery cultures. For example, compare the morphology of HW tumor (fig. 1) with the HW culture (fig. 4).

Description of Recovery Cultures

Strain HW.—Two days after explantation, there was a uniform, thin fringe of cells around the implant (fig. 2). By the 10th day *in vitro*, many of the cells had become rounded, others showed extreme cytoplasmic granulation or vacuolization, while the debris of disintegrated cells was scattered among the remaining healthy cells (fig. 3). This degeneration of the outgrowing cells continued for several days, with new cells migrating from the explant and overgrowing the dead ones. After 28 days, the outgrowth from the clump had increased somewhat in density, but was still not luxuriant.

In succeeding culture generations the outgrowth of the cells was increasingly dense and healthy. The cells in the fringe of outgrowth were elongate in shape and tended to cohere laterally into slender strands. The terminal processes of the cells were threadlike, and the edges of the cell body and of the processes often showed marked rippling (fig. 4). Cell size was uniform. Mitotic activity was high.

Strain D.—The cells in the healthy dense fringe, which had grown out from the explant after 2 days, were comparable in size with those of strain HW at the same time. There was only slight evidence of disintegration of the cells after 10 days' growth *in vitro* (fig. 5). Rather, the cells continued to migrate out and proliferate so rapidly that after 28 days the strain was well established and growing luxuriantly. The cells were for the most part spindle shaped, uniform in size, and were loosely coherent into slender, interlacing or interconnecting strands. The cytoplasm was optically clear, and mitoses were frequent (fig. 6).

Strain D had apparently stabilized in morphology by the end of the 1st culture generation, since there was little variation in morphology in the succeeding generations.

Strain H.—The growth of strain H was very poor during the whole of the first generation. Two days after explantation, the scanty outgrowth of cells formed short, stiff strands with little tendency to interlace or interconnect. After 10 days, the zone of cell outgrowth was still narrow and of low cell density. Many disintegrating cells, characterized by granular and vacuolated cytoplasm, were interspersed between the living cells of the outgrowth (fig. 7). Even by 28 days there was no recovery of a healthy cell outgrowth, and the cultures were made up almost entirely of spherical cells with extremely granular cytoplasm.

In the 2d generation the outgrowth was not luxuriant, but the cells were no longer disintegrating. Strain H stabilized morphologically in

929548—51——18

the succeeding generations and was typified by cells that were coherent into rather long, stiff strands (fig. 8). The elongated cells were uniform in size, showed no abnormal granularity in the cytoplasm, and showed high mitotic activity.

Strain J.—Very few cells migrated from the explant in most of the cultures of strain J after two days *in vitro*. Where migration had occurred, the cells were so closely packed together that cell boundaries were difficult to distinguish (fig. 9). At 10 days after explantation there was distinct morphologic variability in the cultures. One culture out of the 10 had a growing fringe made up of fibroblast-like cells which showed little granulation and were arranged in a loose network. In the remaining cultures a profusion of discrete spindle-shaped cells, with granular cytoplasm and rippling, amoeboid, cytoplasmic periphery, had formed a loose halo around the original explant. Interspersed among them were a few strikingly large, multinucleated cells with heavily granular cytoplasm. These large, oval cells measured up to 300 μ in diameter, and a great part of their volume was occupied by numerous nuclei, each of which contained 1 or 2 conspicuous nucleoli (fig. 10). These large cells were completely overgrown by the smaller cells after 28 days of cultivation. The single culture with cells of the fibroblast-like type grew well and maintained its morphologic individuality.

In the 2d generation, also, there was a lack of morphologic uniformity between the individual cultures. In many of them, the cell outgrowth consisted almost entirely of extremely large mononucleate (and occasionally binucleate) flattened cells which often cohered into an uninterrupted sheet. The free surface of the cell sheet was either lobed and ruffled as in epithelial cultures, or was more irregular and broken with lateral and terminal processes extending from the cells into the clot. The individual cells contained proportionately large nuclei, some of them measuring as much as 45 μ in diameter, while the nucleoli measured up to 10 μ in diameter. The cytoplasm was heavily granular in the region around the nucleus but was thin and transparent at the edges of the cell (fig. 11).

In the remaining cultures, the growing fringe was largely made up of normal-sized, fibroblast-like cells with occasional isolated giant cells growing among them.

No stable morphology was ever obtained in the cultures of this strain. In the 3d generation, some cultures were composed almost entirely of sheets of the large mononucleate and binucleate cells, while in other cultures the growing fringe was made up of strands of cells that occasionally interconnected but usually appeared as stiff rods of nearly uniform diameter. The constituent cells of these strands were in the size-range of the fibroblast, were unusually wide and short, and were closely coherent (fig. 12). The cytoplasm of the cells was granular. Mitoses were observed among these smaller cells, but only rarely were the large cells seen in cleavage.

The failure of the large cells to divide rapidly may explain their increasing scarcity in later culture generations and the tendency for the cultures to be composed largely of cells within the normal range of size for the fibroblast. The cytoplasm of most of these smaller cells was granular, and the cytoplasmic processes were short. Mitotic activity even in the smaller cells was not high.

The fact that the large cells were characteristic of recovery strain J was confirmed by their presence in the three additional series of cultures explanted from strain J tumors. Photomicrographs of these later recovery cultures taken at 15 days in the 2d generation *in vitro* showed the same morphologic design of the cells and the same structure of the cultures observed in the first series of explants.

Strain L.—Two days after explantation, the cultures of strain L were showing a relatively wide and densely cellular fringe of slightly granular but healthy appearing cells. After 10 days the closely coherent cell sheet had broken up into a loose jumble of discrete cells with heavily granular cytoplasm (fig. 13), but by 28 days a new wave of cellular outgrowth had reassumed a relatively coherent sheetlike structure.

The morphology of the cultures in the later generations was constant. The cells were a little smaller than those of the 2d generation and were coherent in a thin sheet. The body of the sheet was made up of somewhat elongated cells, and the edges of the sheet were broken up into short, jagged cell strands that interconnected with one another (fig. 14). The cell strands were composed of elongated, polygonal cells, and the peculiarly jagged appearance of the strands was due partly to the processes projecting from the corners of these cells and partly to the erratic manner in which these cells connected with one another. There was little cytoplasmic granulation, and mitoses were numerous.

Strain N.—The cells of strain N formed a wide dense fringe around the explant after 2 days of cultivation *in vitro*. After 10 days *in vitro*, there occurred the same disintegration of the growing fringe, with the same formation of discrete, heavily granular cells that was observed in strain L. In addition, slender strands of elongated spindle-shaped cells radiated out from the explant between the loose granular cells to join, on occasion, small clusters of flattened, closely coherent, and slightly granular cells (fig. 15).

By 28 days, a large proportion of the cells had assumed rounded or spherical shapes. Close to the explant in some cultures, there was general necrosis and disintegration of the cells, but at the periphery there were still small areas of flattened, slightly granular cells, which seemed healthy.

The cells of strain N never became adapted to culture conditions. Immediately after explantation in later generations, the cultures showed a rapid outgrowth of a thick massive sheet of closely coherent cells (fig. 16), comparable in design to that seen in the original strain N cultures, but within a few days after the appearance of the outgrowth, shrinkage, necrosis, and disintegration of the cells occurred in the zone between the

explant and the periphery of the fringe. The necrosis would progress until, after 15 days, nests of closely coherent cells, which originally formed the extreme edge of the culture, were separated from the explant and from each other by wide areas of degenerating and necrotic cells.

Although every effort was made to maintain the cultures, the rate of division of the healthy cells failed to compensate for cell disintegration, so that the cell population of the cultures became progressively smaller. At the end of the 4th generation, when it became clear that the strain could not be maintained, the cultures were injected into mice.

Strain O.—The cultures of strain O at 2 days after explantation showed only small areas of cell migration and proliferation (fig. 17). At 10 days, liquefaction of the clot occurred around the central explant and in each culture the explant soon sloughed out. These cultures were patched with plasma, and the sparse growth that occurred at 28 days was considered to be atypical because of this manipulation. At 13 days in the 2d generation, growth was still very limited and consisted of a narrow, dense fringe of cells with loose, rounded, isolated cells at the periphery (fig. 18).

In the 3d generation, the cell fringe was a massive sheet of closely coherent cells (fig. 19). The free edges of the fringe were either quite smooth, or slightly lobed, but showed no extended strands. The free edges of the peripheral cells were either slightly rippling or entirely smooth, and because of the thickness of the cell sheet, nuclear structure could not be ascertained. One culture deviated from this architecture by showing a loose, interlacing network of laterally discrete cells, ranging from spindle to elongate polygonal shapes.

The morphology of the cells in the succeeding generations followed a rather consistent pattern. The cell fringe no longer consisted of the massive sheets that occurred in the 3d generation. The edge of the fringe was broken up into rather slender strands. The cells of these strands varied from a spindle-shaped cell, with an extraordinary degree of rippling at the edges, to a short, equidimensional polygonal-shaped cell (fig. 20). Cytoplasmic granularity of the cells varied from practically none, in some areas, to extreme granularity in others. There was considerable range of cell size, but no extremely large cells were seen. Mitoses were frequent.

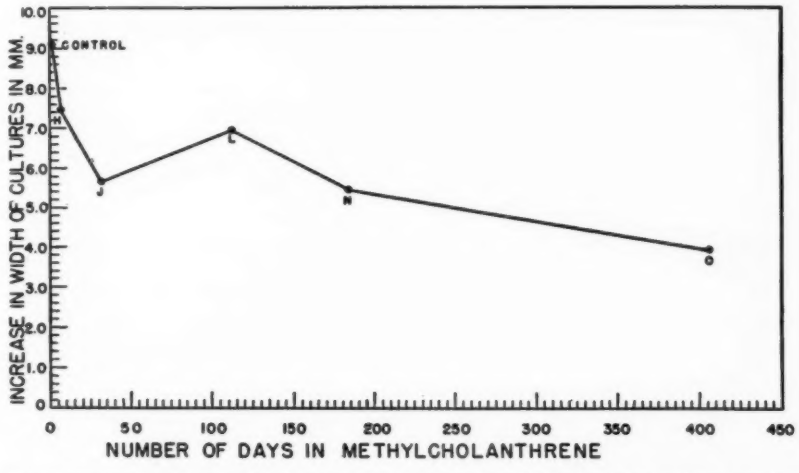
Comparison With Original Cultures

Since the original HW strain was closed by injection into mice in 1942, comparison of the recovery strain with the original strain was not possible. The morphology of recovery strain HW, however, was similar to that of the other untreated cell strain D. With the exception of strain J, the morphology of the cells of the other recovery cultures reproduced in remarkable detail the morphology of the parent cultures (*1*). The degree of progressive morphologic deviation from the normal pattern of the cultured fibroblast observed in the stabilized recovery cultures was of the same order as the changes present in the original cell strains. Recovery strains D and H had certain characteristics in common, and the similarity between these two strains was also observed in the original cultures. Re-

covery strain L was distinctly different in architecture from recovery strains HW, D, and H. The cells were less spindle-shaped, more sheet-like, had shorter terminal processes, and tended to form a more closely coherent sheet than did the cells of the preceding strains. This morphology was similar to the design of the original strain L cultures. The morphology of recovery strain N indicated an even greater alteration from the normal fibroblast than did recovery strain L, in that the cells were heavily granular, and were packed together in a sheet which was extended at the edges into lobes. This was a degree of alteration similar to that of the original N strain. Recovery strain O showed two types of morphology. Some cultures grew as a closely packed, smooth-edged, massive sheet of very granular cells, while in others, short strands of shrunken and rounded cells projected from the edge of the outgrowth. Each of these types was also characteristic for the original O strain.

DISCUSSION

One of the roughly defined characteristics of the *original* cultures of these cell strains was the rate at which the cellular outgrowth migrated from the central implant. As a general rule, the cellular outgrowth of the original strains D, H, and L was wider than that of the original strains J, N, and O, and the plot of the culture widths against the time that each strain was exposed to the carcinogen produced the shape of curve seen in text-figure 2. When the widths of the recovery cultures were plotted in like fashion, a curve with a similar shape resulted (text-fig. 1). While the absolute values for the widths of the original cultures and the recovery cultures do not correspond, the real interest in these curves lies in the fact



TEXT-FIGURE 2.—Curve showing average increase in width of *original* culture strains plotted against time in carcinogen. The control value represents an average of all control cultures through September 1942. The other values represent averages of culture widths through May 1943.

that, so far as the relation of the strains to each other is concerned, the degree to which the cells migrated from the explant was a stable characteristic unaffected by the period of growth in the animal. The curves, unfortunately, do not lend themselves to further interpretation. More significant results could be obtained in future studies of this type by using the newly developed methods for estimating the size of the cell population of tissue cultures (*4*), or by using comparison cinematographic methods for recording rates of cell migration.

Before using culture morphology as a means of comparison of the original and recovery strains it was necessary to wait until the latter had established a stable culture pattern. The initial cellular outgrowth in the recovery cultures was an unstable system that required a considerable period of time to reach an equilibrium condition where the morphologic identity of the culture strains became recognizable. Only after the recovery cultures became stabilized could it be shown that their morphology (strain J excepted) was not materially affected by the period of growth *in vivo*. With the exception of strain J, the morphology of each of the other strains closely resembled that of its corresponding original culture.

The morphologic pattern of strain J was dissimilar to that of the original cultures, and it seems possible that in the case of this strain there was a host influence, either upon the tumor cells themselves, or upon the cell population of the tumor. The inability of the recovery strain J cells to produce more than 1 tumor out of 97 injections supports the latter possibility. The tumor itself was readily transplantable from animal to animal, and with the exception noted at the beginning of this paper, always with 100 percent "takes." The loss of this tumor-producing capacity by the tumor cells in culture cannot, of course, be satisfactorily explained, but it brings up some interesting questions. Would the cultures have produced tumors in the animals if they had been injected into mice after 30 days *in vitro* instead of after longer intervals in culture? In the first series of explantations from the J tumor, the cultures were not injected into mice until 155 days had passed. No tumors were ever produced from this series. But in the second series of explantations from the J tumors, the recovery cultures were injected into mice after only 79 days *in vitro*, and 1 tumor resulted from a total of 34 injections. This is some indication that more tumors might have been produced by the injection of cultures with an even shorter *in vitro* life-span.

The corollary to the above consideration is the question of whether, with continued life *in vitro*, there occurred in this strain a progressive selection of cells that were incapable of producing tumors when injected into mice. It seems more probable that something of this nature occurred rather than an alteration in the cells themselves, since tissues from the other 6 tumors were capable of producing tumors after long periods in culture. Further work will be necessary before these questions can be answered.

It was of interest that the histologic appearance of the tumors gave little indication as to what the resulting *in vitro* morphologic pattern of the

cell strains would be. This was particularly true of recovery strain J. No giant cells with polyploid nuclei were found in the sections of the tumors used for culture, but the appearance of large mononucleate and binucleate cells in 4 series of explantations from 4 separate strain J tumors indicated that the presence of these cells was not a chance phenomenon, but a consistent characteristic of the strain J tumor grown *in vitro*.

The behavior of the freshly explanted tumor tissues during the first 28 days in culture as compared with their behavior in subsequent generations demonstrates the instability of these freshly explanted tissues. Following explantation, all of the tissues with the exception of strain D underwent a series of degenerative phenomena characterized by extreme cytoplasmic granularity, separation of the coherent cell sheets into discrete cells, and disintegration and death of these individual cells. Recovery from this condition was fast or slow, depending upon the strain. The final establishment of a relatively stable culture morphology was not accomplished at the same rate by the tissues of the different sarcomas. It consumed from 28 days in the case of strain D, to 100 days in the case of strain O, or, as in the case of strain N, the cells never became adapted to culture conditions.

In these studies, a heterologous medium was used that was designed to promote rapid cell proliferation, and the aim was to produce maximum cell growth rather than to maintain a close structural relationship of viable cells to each other. In explanting tissue from these sarcomas, a heterogeneous population of cells was introduced into foreign surroundings, and since the culture medium used did not reproduce the milieu of the host animal, certain adjustments must have been made by the tissue if it was to survive in culture for any length of time. These adjustments probably involved the selective death of certain cell types incapable of survival, and certain adaptations by the cells that did survive, which permitted them to live and grow. The work of Sanford *et al.* (*5*) indicates that this cell adaptation may involve a conditioning of the medium itself, since they were able to propagate cultures from single cells only after using a medium in which cells had previously grown.

The nature of the adjustments made by tissues to culture conditions has yet to be fully explored. The variations found in the explants of these tumors indicate clearly the need for further definitive experiments on the physiology of freshly explanted tissue.

SUMMARY

Recovery cultures were set up by explanting tissue from 7 sarcomas which, in turn, had been produced by the injection of 7 tissue-culture strains (original cultures) into C3H mice. Comparisons of growth characteristics and morphology were made between the recovery cultures and the original cultures.

When increase in culture width was used as a criterion of comparison, it was found that the recovery cultures preserved the essential relationship

to each other that had been demonstrated for the original cultures (*cf.* text-figs. 1 and 2).

With the exception of strain J, the morphology of the recovery cultures closely resembled that of the original cultures. The morphology of recovery strain J was unlike that of the original strain J. This suggests some influence of the animal host upon this strain.

When the tumor tissues were explanted, the first cell outgrowth appeared healthy, but after a few days in culture many of the cells became rounded, vacuolated, or heavily granular, and many died. Recovery from this phase of instability to an equilibrium condition of stable morphology proceeded at different rates for the different cell strains. The need for further definitive study of the physiology of freshly explanted tissue is indicated.

REFERENCES

(*1*) EARLE, W. R.: Production of malignancy *in vitro*. IV. The mouse fibroblast cultures and changes seen in the living cells. J. Nat. Cancer Inst. 4: 165–212 (1943).

(*2*) EARLE, W. R., and NETTLESHIP, A.: Production of malignancy *in vitro*. V. Results of injection of cultures into mice. J. Nat. Cancer Inst. 4: 213–228 (1943).

(*3*) EARLE, W. R., SCHILLING, E. L., and SHELTON, E.: Production of malignancy *in vitro*. IX. Description of cells at the fluid interface of the culture. J. Nat. Cancer Inst. 10: 865–871 (1950).

(*4*) SANFORD, K. K., EARLE, W. R., EVANS, V. J., WALTZ, H. K., and SHANNON, J. E.: The measurement of proliferation in tissue cultures by enumeration of cell nuclei. J. Nat. Cancer Inst. 11:773–796 (1951).

(*5*) SANFORD, K. K., EARLE, W. R., and LIKELY, G.: The growth *in vitro* of single isolated tissue cells. J. Nat. Cancer Inst. 9: 229–246 (1948).

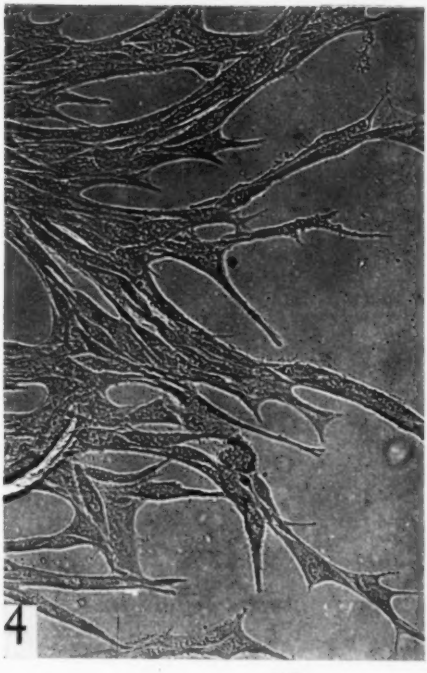
PLATE 85

FIGURE 1.—Tumor strain HW, from which recovery culture strain HW originated. Compare with cells in culture. Hematoxylin-eosin stain. × 400.

FIGURE 2.—Recovery strain HW, 2 days after explantation cells appear healthy. × 200.

FIGURE 3.—Recovery strain HW, 10 days after explantation. Note cellular debris. Many cells appear to be dying. × 200.

FIGURE 4.—Recovery strain HW. Stabilized morphology. × 200.



Shelton and Earle

929548—51——19

PLATE 86

FIGURE 5.—Recovery strain D, 10 days after explantation. Only slight evidence of cell disintegration or death. × 200.

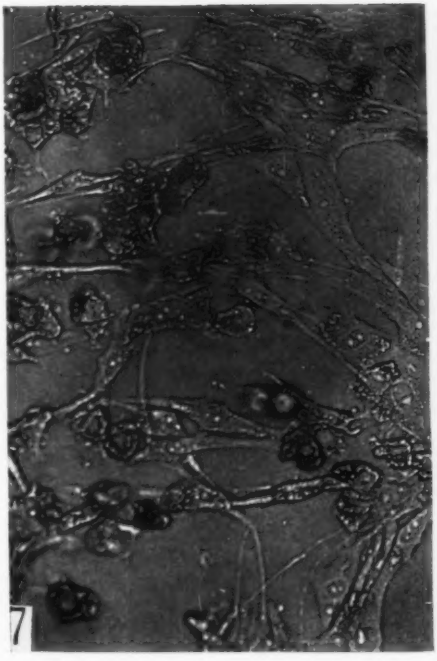
FIGURE 6. Recovery strain D. Stabilized morphology. × 200.

FIGURE 7.—Recovery strain H, 10 days after explantation. Many cells are rounded. × 200.

FIGURE 8.—Recovery strain H, stabilized morphology. × 200.

5

6


7

8

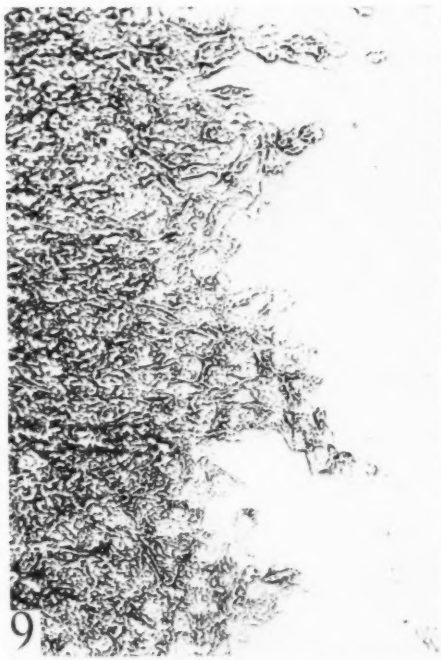
PLATE 87

FIGURE 9.—Recovery strain J, 2 days after explantation. Apparently healthy cells are in closely packed sheet. × 200.

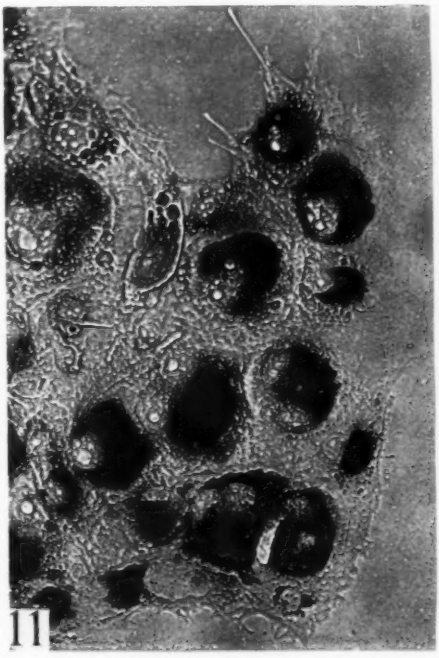
FIGURE 10.—Recovery strain J, 10 days after explantation. Cells are disoriented and heavily granular. Note multinucleated cell. × 200.

FIGURE 11.—Recovery strain J, 69 days after explantation, 3d generation. Culture composed almost exclusively of very large cells with densely granular cytoplasm. × 200.

FIGURE 12.—Recovery strain J, 69 days after explantation, 3d generation. Cells of this culture approach size-range of fibroblast. × 200.


9

10


11

12

PLATE 88

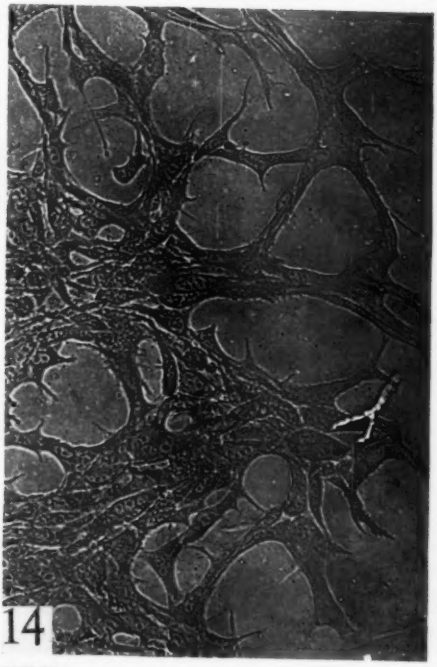
FIGURE 13.—Recovery strain L, 10 days after explantation. Outgrowth has lost all semblance of a cell sheet. Some cells rounded up, many have extremely opaque cytoplasm. × 200

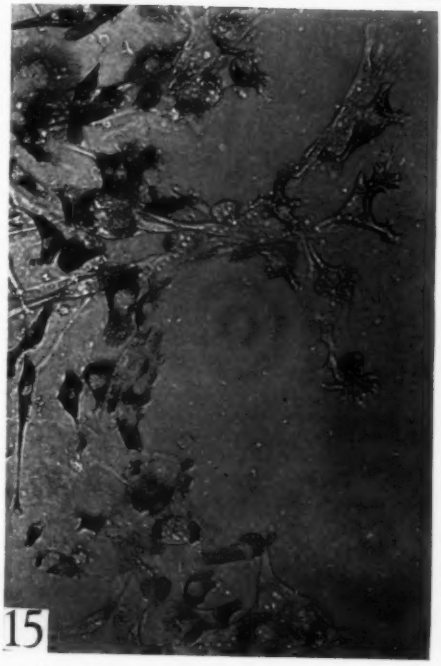
FIGURE 14.—Recovery strain L. Stabilized morphology. × 200.

FIGURE 15.—Recovery strain N, 10 days after explantation. Note increased granulation of cytoplasm and evidence of disintegration of some cells. × 200

FIGURE 16.—Recovery strain N, 95 days after explantation, 4th generation. This coherent cell sheet later broke down into separated cells. × 200.

13


14


15

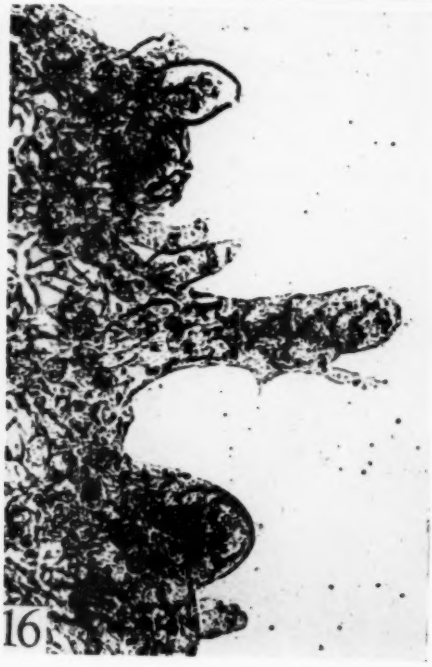

16

PLATE 89

FIGURE 17.—Recovery strain O, 2 days after explantation. Outgrowth very limited. Cells have granular cytoplasm. × 200.

FIGURE 18.—Recovery strain O, 41 days after explantation. Cells of this strain still show the rounding and disorientation characteristic of an unstabilized culture. × 200.

FIGURE 19.—Recovery strain O, 3d generation. Some culture showed massive cell sheets. Compare with Earle's photograph of the original strain (*1*). × 200.

FIGURE 20.—Recovery strain O, 4th generation. Stabilized morphology. × 200.

929548—51——20

THE EFFECT OF PODOPHYLLOTOXIN ON THE SKIN OF THE MOUSE, WITH REFERENCE TO CARCINOGENIC, COCARCINOGENIC, AND ANTICARCINOGENIC ACTION [1]

I. BERENBLUM,[2] *Special Research Fellow, National Cancer Institute, National Institutes of Health, U. S. Public Health Service, Bethesda, Md.*

Recent interest in podophyllin—the alcohol-soluble resin of the mandrake, or May apple—arose out of the effectiveness of the resin itself and one of its constituents, podophyllotoxin, in the topical treatment of condyloma acuminatum (*2*, *8*), their colchicine-like action on skin epithelium (*3*, *4*), and on cells in tissue culture (*6*), and the pronounced inhibitory effect of the resin and several of its isolated constituents on the growth of transplanted tumors in animals (*1*, *5*, *7*).

This communication is concerned with tests for carcinogenic, cocarcinogenic, and anticarcinogenic action of podophyllotoxin, carried out by painting experiments on the skin of the mouse.

MATERIALS AND METHODS

The podophyllotoxin, isolated from commercial podophyllin by Dr. J. L. Hartwell and Mr. W. E. Detty [batch D 242—previously used by Leiter *et al.* (*5*) for tumor-damaging tests], was made up as a 2.5-percent solution in polyethylene glycol-400, using the solvent alone for the control group. Both of these were applied with a glass rod to an area of skin, about 1.5 cm. square, in the dorsal region over the shoulderblades. In the tests for cocarcinogenic and anticarcinogenic action, the carcinogen used was a 0.5-percent solution of 3,4-benzpyrene (Edcan Laboratories) in acetone, one drop being administered to the area of skin from a Pasteur pipette. In all cases, the hair over the area of skin was removed previous to each painting by clipping with fine scissors.

The mice used in these experiments were C3H virgin females, about 2 to 3 months old at the start, obtained from the National Cancer Institute colony. They were kept in plastic cages, 25 per cage, and fed Purina laboratory chow, supplemented once weekly with raw carrot, and water

[1] Received for publication November 14, 1950.

[2] Present address: Department of Experimental Biology, Weizmann Institute of Science, Rehovoth, Israel.

929548—51——21

ad libitum. The animal room in which they were kept was air-conditioned and maintained at a temperature of 74°–80° F.

All induced tumors were charted when first observed, and regularly at 2-week intervals thereafter, careful note being taken of the time of appearance of the first induced tumor per animal, and the time when malignancy supervened, as judged by naked-eye evidence. After death, the lesions were examined microscopically for confirmation of malignancy. Many of the animals also developed mammary tumors, but these were deemed to have arisen spontaneously, and are not taken into account here.

RESULTS

Test for Carcinogenic Action

Twenty-five mice were painted twice weekly for 74 weeks with a 2.5-percent solution of podophyllotoxin in polyethylene glycol-400. The animals survived the treatment well, no deaths occurring in the first 29 weeks of treatment. After the 50th week, there were still 20 survivors; 5 of these were killed for histologic study, and of the remaining 15 mice, 13 were still alive at the end of the experiment (74th week of painting).

The reaction of the skin to the repeated treatment was much milder than that observed after a single painting, thus confirming the observation of King and Cauldwell (*3*) of an adaptation of the mouse's skin to the action of this drug. "Podophyllin cells" were rarely seen after the repeated treatment, the general histologic picture being that of a mild hyperplastic response of the epithelium with some atrophic changes, not noticeably different from the characteristic skin response to repeated mild irritation of a nonspecific kind.

No skin tumors developed in any of the treated animals at the site of application.

Test for Cocarcinogenic or Anticarcinogenic Action

By applying the drug concurrently with a carcinogen acting under suboptimal conditions, it was possible to test for cocarcinogenic and anticarcinogenic action at the same time. A shortening of the latent period, in comparison with the control series, would indicate cocarcinogenic action; a lengthening would indicate anticarcinogenic action.

One hundred mice, divided into 2 equal groups, received weekly skin applications of 0.5 percent benzpyrene in acetone. One group received, in addition, weekly application, alternating with the benzpyrene treatment at a 3- to 4-day interval, of 2.5 percent podophyllotoxin in polyethylene glycol, while the other group, serving as a control, received the solvent (polyethylene glycol) without podophyllotoxin. In both, the treatment was continued for 52 weeks.

As shown in table 1, skin tumors developed in 43 out of 47 survivors of the control series (representing 91 percent), but only in 28 out of 44 survivors in the experimental group (representing 64 percent). In both series, over 50 percent of the tumors ultimately became malignant. The average latent period for total skin tumors (*i.e.*, the time for 50 percent of

survivors to develop tumors), was 42 weeks in the control group, and 48 weeks in the experimental group, thus representing a delay of 6 weeks when podophyllotoxin supplemented the benzpyrene treatment.

TABLE 1.—*Effect of podophyllotoxin on skin carcinogenesis induced by 3, 4-benzpyrene*

	Benzpyrene control	Benzpyrene plus podophyllotoxin
Number of animals used	50	50.
Effective total (survivors at time when first tumor appeared in group)	47	44.
Time of appearance of first tumor	23d week	29th week.
Time of appearance of first malignant tumor	29th week	43d week.
Total number of tumor-bearing animals	43	28.
Percentage of effective total bearing tumors	91 percent	64 percent.
Total number of malignant tumor-bearing animals	34	16.
Percentage of effective total bearing malignant tumors.	72 percent	36 percent.
Percentage of malignant tumors per total tumor-bearing animals.	79 percent	57 percent.
Average latent period for total tumors (=time of appearance of tumors in 50 percent of effective total).	42d week	48th week.

NOTE.—The term 'total tumors' in this table refers to all tumors—papillomas and carcinomas—at the site of application.

In brief, podophyllotoxin, under the conditions of the present experiment, showed no cocarcinogenic effect, but, on the contrary, a slight anticarcinogenic effect.

SUMMARY

Podophyllotoxin, in the form of a 2.5 percent solution in polyethylene glycol-400, was tested on the skin of C3H virgin female mice for carcinogenic, cocarcinogenic, and anticarcinogenic action.

It was found to have no carcinogenic or cocarcinogenic action, but, on the contrary, a slight anticarcinogenic action.

REFERENCES

(*1*) BELKIN, M.: Effect of podophyllin on transplanted mouse tumors. J. Pharmacol. & Exper. Therap. 93: 18–25 (1948).

(*2*) KAPLAN, I. W.: Condylomata acuminata. New Orleans M. & S. J. 94: 388–390 (1942).

(*3*) KING, L. S., and CAULDWELL, E. W.: Effect of podophyllin on mouse skin. II. Consideration of some functional aspects. J. Nat. Cancer Inst. 10: 131–146 (1949).

(*4*) KING, L. S., and SULLIVAN, M.: Effect of podophyllin and of colchicine on normal skin, on condyloma acuminatum, and on verruca vulgaris. Arch. Path. 43: 374–386 (1947).

(*5*) LEITER, J., DOWNING, V., HARTWELL, J. L., and SHEAR, M. J.: Damage induced in sarcoma 37 with podophyllin, podophyllotoxin, alpha-peltatin, beta-peltatin, and quercetin. J. Nat. Cancer Inst. 10: 1273–1293 (1950).

(*6*) ORMSBEE, R. A., and CORNMAN, I.: The effect of podophyllin on tumor cells *in vitro*. (Abstract) Cancer Research 7: 717 (1947).

(*7*) STOCK, C. C., SUGIURA, K., and RHOADS. C. P.: Effect of metabolic products of fungi on the viability and growth of carcinoma, sarcoma and melanoma in mice. (Abstract) Cancer Research 8: 384 (1948).

(*8*) SULLIVAN, M., FRIEDMAN, M., and HEARIN, J. T.: Treatment of condylomata acuminata with podophyllotoxin. South. M. J. 41: 336–337 (1948).

THE ACTION OF CROTON OIL IN THE INDUCTION OF SARCOMAS IN MICE[1,2]

MICHAEL KLEIN, *Research Fellow, National Cancer Institute, National Institutes of Health, U. S. Public Health Service, Bethesda, Md.*

It has been suggested that at least two major phases are involved in skin tumorigenesis (*1*, *2*). On the one hand, there is the initiation of the neoplastic change and on the other, the promotion of altered cells to the visible tumor stage. Berenblum (*3*) observed that croton oil, which of itself produced much hyperplasia but no neoplasms, had a marked effect on the development of skin tumors in mice when applied with 3, 4-benzpyrene in acetone. Mottram (*4*) demonstrated that one application of a carcinogen was incapable of eliciting skin tumors by itself but did so when followed by paintings with croton oil. In this way, the promoting action of the latter chemical was further elucidated. Berenblum and Shubik (*5*, *6*) confirmed these results and observed further that a lapse of many weeks between the application of a carcinogen and the start of croton-oil paintings did not affect the total tumor yield. The cocarcinogenic action of croton oil appeared specific for the skin of mice, however, for no such effect was observed in rats, rabbits, or guinea pigs (*7*, *8*). In an attempt to demonstrate cocarcinogenesis at a different site, croton resin was injected with 3, 4-benzpyrene subcutaneously into rats (*7*). No promoting action was observed. However, Sall and Shear (*9*) previously reported that the subcutaneous injection of a minimal dose of 3, 4-benzpyrene with the basic fraction of creosote oil into strain A mice resulted in more tumors, and these appeared sooner than those induced by the carcinogen alone. The following experiments were designed to investigate the action of croton oil in the presence of 20-methylcholanthrene upon tumor formation in mice.

MATERIALS AND METHODS

The animals used in these experiments were male albinos derived from a colony of originally strain A mice in which the agouti gene appeared, indicating a possible outcrossing to another albino strain, probably C. Animals were housed in plastic cages measuring 27.5 by 17.5 by 12.5 cm., generally 8 to a cage. They were maintained on pellets of Purina laboratory chow and had access to a supply of tap water. Young adult males

[1] Received for publication November 17, 1950.
[2] Acknowledgment is made of the technical assistance of Mr. Frank M. McCathran.

3 to 5 months of age were injected once intramuscularly in a hind limb with one of the following solutions:

Series A.—One percent croton oil[3] in olive oil (U. S. P. grade).

Series B.—Olive oil, containing 0.6 mg. of methylcholanthrene[4] per cc.

Series C.—One percent croton oil in olive oil, containing 0.6 mg. of methylcholanthrene per cc.

Series D.—Olive oil, containing 0.1 mg. of methylcholanthrene per cc.

Series E.—One percent croton oil in olive oil, containing 0.1 mg. of methylcholanthrene per cc.

Solutions were injected with a 1/4-cc. tuberculin syringe and an 18-gauge short-beveled needle. The skin around the point of entrance of the needle was pinched off for several seconds. Animals received 0.04 cc. of one of the above solutions together with 0.08 cc. of Locke's solution per injection. Thus, each mouse in Series A was injected with 0.0004 cc. of croton oil; in Series B, with 0.024 mg. of methylcholanthrene; in Series C, with 0.0004 cc. of croton oil and 0.024 mg. of methylcholanthrene; in Series D, with 0.004 mg. of methylcholanthrene; and in Series E, with 0.004 mg. of methylcholanthrene and 0.0004 cc. of croton oil.

At autopsy, tissues were excised and fixed in Tellyesniczky's fluid (2 parts formol, 20 parts 70 percent alcohol, and 1 part acetic acid). Tissues were dehydrated, imbedded in paraffin, sectioned at 6 to 8 micra, and stained with hematoxylin and eosin. Diagnoses[5] were based on histologic findings. The latent period of tumorigenesis was calculated from the time that tumors first became palpable.

OBSERVATIONS

The control mice in Series A (table 1) each received one injection of a 1-percent solution of croton oil in olive oil. None of the 33 mice in the group showed signs of a tumor, although 17 lived as long as 479 days, when the experiment was terminated. Of the 40 mice in Series B, each of which received 0.024 mg. of methylcholanthrene in olive oil, 20 bore tumors. On the average, these neoplasms were detected after 160.1 days. The mean age of all mice in Series B at death was 268.3 days. Each of the 40 animals in Series C also received 0.024 mg. of methylcholanthrene. However, the carcinogen now was administered in a solution of croton oil in olive oil. A 100-percent tumor incidence was observed. The tumors in Series C were detected after a mean latent period of 95.4 days. The mean age at death for mice in this group was 116 days. When the concentration of methylcholanthrene in olive oil was decreased to 0.1 mg. per cc. (Series D), 9 out of 32 mice had tumors. These were first recorded after a mean latent period of 168 days. The average age at death for all the mice in Series D was 298.9 days. The same concentration of methylcholanthrene was employed in Series E as in Series D, except that in Series E the carcinogen was dissolved in a solution of croton oil in olive

[3] Obtained from the Fischer Scientific Company, Eimer and Amend, New York, N. Y.

[4] Obtained from Edcan Laboratories, South Norwalk, Conn. MP 179–180°C.

[5] The author wishes to thank Dr. Thelma B. Dunn of the Pathology Section of the National Cancer Institute for her assistance.

TABLE 1.—*Intramuscular injection of methylcholanthrene and croton oil in albino mice*

Series	Treatment			Mice in experiment	Results					Average age at death, all mice
	Solution and concentration	Methylcholanthrene per mouse	Croton oil per mouse		Mice with tumors	Tumor incidence	Significance of difference [1]	Mean latent period	Significance of difference of means	
		Mg.	*Cc.*	*Number*	*Number*	*Percent*		*Days*		*Days*
A	1 percent croton oil in olive oil		0.0004	33	0					445.5
B	0.6 mg. methylcholanthrene per cc. of olive oil.	0.024		40	20	50.0	$t=5.16$ and $p=<.01$	160.1	$t=3.95$ and $p=<.01$	268.3
C	0.6 mg. methylcholanthrene per cc. of a 1 percent solution of croton oil in olive oil.	.024	.0004	40	40	100.0		95.4		116
D	0.1 mg. methylcholanthrene per cc. of olive oil.	.004		32	9	28.1	$t=4.6$ and $p=<.01$	168	$t=5.12$ and $p=<.01$	298.9
E	0.1 mg. methylcholanthrene per cc. of a 1 percent solution of croton oil in olive oil.	.004	.0004	32	22	68.7		123.4		202.6

[1] $t=\frac{p_1-p_2}{\sqrt{2\frac{\hat{p}\,\hat{q}}{n}}}$, where p_1=first percentage; p_2=second percentage; $\hat{p}$ =estimate of the "population" percent, assuming no difference; $\hat{q}=1-\hat{p}$; n=number in each separate sample.

oil. Again croton oil accelerated tumor formation and increased the incidence. Thus among 32 mice, 22 bore tumors after a mean latent period of 123.4 days. The mean age at death for mice in Series E was 202.6 days.

Tumors were permitted generally to enlarge to two or more centimeters before a host was sacrificed. Twenty-seven of these tumors were selected at random for transplantation to mice of the same derivation. A 90-percent incidence of takes was observed.

Histologic examination showed all the tumors to be fibrosarcomas. No differences were observed between those induced with methylcholanthrene in olive oil and those obtained following the injection of this carcinogen in a solution of croton oil in olive oil.

DISCUSSION

A significant promoting effect was elicited when methylcholanthrene dissolved in a croton-oil-in-olive-oil solution was injected intramuscularly into albino mice. The incidence of tumors was increased and the latent period of tumorigenesis decreased in the croton-oil series as compared with the series in which methylcholanthrene in olive oil was injected. This was observed at concentrations of both 0.6 mg. and 0.1 mg. per cc. of the carcinogen (table 1). Previously, it had been reported (*7*) that when rats were injected subcutaneously with benzpyrene and croton resin, the active constituent of croton oil, tumor incidence, and latent period remained unaffected. It is to be noted, however, that croton oil has not been shown to exert a promoting action in rats even upon percutaneous administration with a carcinogen (*8*). On the other hand, Sall and Shear (*9*) employing mice, a species known to be susceptible to the cocarcinogenic action of croton oil when applied to the skin, observed a decided promoting action when the noncarcinogenic basic fraction of creosote oil was injected subcutaneously with benzpyrene. They obtained more tumors in less time with the use of creosote oil than when benzpyrene alone was employed.

The tumors observed in mice that had received methylcholanthrene or methylcholanthrene and croton oil (table 1) were fibrosarcomas. Thus it appears that in the induction of mouse tumors croton oil is able to exert a promoting action, not only upon a tissue derived from ectoderm, but also one from mesoderm. Berenblum (*3*) has shown that croton oil appears to play a specific role in skin tumorigenesis that is not related to its activity as an irritant. Thus, turpentine or xylene administered to mice in doses that produced about as much skin irritation as croton oil still exerted no promoting action in papilloma formation. In addition, Beck (*10*) observed that the induction of sarcomas with 3,4-benzpyrene was not altered when the carcinogen was injected subcutaneously with turpentine in olive oil. It was concluded that subacute inflammation caused by the irritant had no effect on the carcinogenic potency of 3,4-benzpyrene. Whether the same factors in croton oil that are responsible for

its promoting action in skin tumorigenesis also operate intramuscularly in the induction of sarcomas is not known. It would appear, however, that a specific role again is indicated for croton oil.

In the induction of skin tumors, one painting with a subminimal dose of a carcinogen followed by repeated application of croton oil elicits many tumors (*5*, *6*). The majority of these are benign papillomas, which proliferate slightly, become keratinized, and are sloughed. Occasionally an epidermal carcinoma is observed. After a sufficient number of paintings with the carcinogen, however, one may either continue with it or substitute croton oil and obtain more such malignant tumors (*11*). These tumors have been observed to arise frequently from sites previously occupied by benign papillomas (*7*, *12*). In addition, Berenblum (*7*) has suggested that in mice, croton resin facilitates the transformation of benign to malignant tumors. In experiments here reported, however, the tumors obtained were all malignant, despite the injection of as little as 0.004 mg. of carcinogen per mouse. Experiments in progress indicate that sarcomas similarly are induced with as little as 0.002 mg. of methylcholanthrene. The fact that these grow rapidly and progressively from the time they first become palpable suggests the malignant change may have been present from the onset.

Susceptibility of the intramuscular tissues of mice to small amounts of a carcinogen in the presence of croton oil may provide a sensitive test for the detection of carcinogens. Smith (*13*) described a similar technique which involved implantation of fetal tissues together with a carcinogen, Scharlach R, and croton oil. It appears that in testing for carcinogenicity the same end may perhaps be attained without the introduction of fetal tissues and Scharlach R.

SUMMARY

Albino mice received intramuscular injections of 0.024 mg. or 0.004 mg. of methylcholanthrene dissolved either in olive oil or in a 1 percent solution of croton oil in olive oil. A higher tumor incidence and a lower latent period were observed in the latter series. Thus, the promoting action of croton oil first revealed in skin tumorigenesis appears also to be involved in the induction of sarcomas. It is suggested that the use of croton oil with the intramuscular-injection technique may be a sensitive test for substances suspected of being carcinogens.

REFERENCES

(*1*) Berenblum, I.: Irritation and carcinogenesis. Arch. Path. 38: 233–244 (1944).

(*2*) Friedewald, W. F., and Rous, P.: The initiating and promoting elements in tumor production. J. Exper. Med. 80: 101–144 (1944).

(*3*) Berenblum, I.: The co-carcinogenic action of croton resin. Cancer Research 1: 44–49 (1941).

(*4*) Mottram, J. C.: A developing factor in experimental blastogenesis. J. Path. & Bact. 56: 181–187 (1944).

(5) BERENBLUM, I., and SHUBIK, P.: The role of croton oil applications associated with a single painting of a carcinogen in tumor induction of the mouse's skin. Brit. J. Cancer 1: 379–382 (1947).

(6) ———: A new, quantitative approach to the study of the stages of chemical carcinogenesis in the mouse's skin. Brit. J. Cancer 1: 383–391 (1947).

(7) BERENBLUM, I.: The mechanism of carcinogenesis. A study of the significance of co-carinogenic action and related phenomena. Cancer Research 1: 807–815 (1941).

(8) SHUBIK, P.: Studies on the promoting phase in the stages of carcinogenesis in mice, rats, rabbits, and guinea pigs. Cancer Research 10: 13–17 (1950).

(9) SALL, R. D., and SHEAR, M. J.: Studies in carcinogenesis. XII. Effect of the basic fraction of creosote oil on the production of tumors in mice by chemical carcinogens. J. Nat. Cancer Inst. 1: 45–55 (1940).

(10) BECK, S.: On the failure of acute and subacute inflammation to influence carcinogenesis with 3:4 benzpyrene. Brit. J. Exper. Path. 19: 319–323 (1938).

(11) RUSCH, H. P., and KLINE, B. E.: Influence of interrupted carcinogenic treatment on tumor formation. Proc. Soc. Exp. Biol. & Med. 69: 90–95 (1948).

(12) MOTTRAM, J. C.: The change from benign to malignant in chemically induced warts in mice. Brit. J. Exper. Path. 26: 1–4 (1945).

(13) SMITH, W. E.: The tissue transplant technic as a means of testing materials for carcinogenic action. Cancer Research 9: 712–723 (1949).

OBSERVATIONS ON PROPERTIES OF LEUKEMIC CELLS RESISTANT TO FOLIC ACID ANTAGONISTS[1]

L. W. LAW, with the technical assistance of Mr. PETER J. BOYLE, *National Cancer Institute, National Institutes of Health, Public Health Service, Bethesda, Md.*

INTRODUCTION

It has been postulated that the resistance developed in bacteria and other microorganisms when they are brought in contact with various antimicrobial agents may be either 1) induced by the agent under investigation or 2) the result of genetic changes, independent of the action of the agent. Appropriate experiments made to determine the manner of action of certain antimicrobial agents indicate that the resistance observed originates through spontaneous genetic changes and that the agents used act in a selective manner, eliminating susceptible forms and allowing resistant individuals to reproduce. Resistance of *Escherichia coli* to bacteriophage (*1*), of *Staphylococcus aureus* to penicillin (*2*, *3*), of *Escherichia coli* to ultraviolet radiation (*4*), and of *Staphylococcus aureus* to a sulfonamide (*5*) has been shown to be due to genetic changes occurring in individual cells of the culture, independent of the action of the agent used.

The development of resistance is not confined to microorganisms. We have reported the development of resistance in mouse leukemic cells, derived from a transplantable acute lymphoid leukemia, to three antileukemic agents: 4-amino, N^{10}-methyl pteroylglutamic acid (amethopterin) 4-amino, 9-methyl pteroylglutamic acid (aninopterin) and 4-amino, 9, N^{10}-dimethyl pteroylglutamic acid (adenopterin)[2] (*6*). All three resistant variants obtained were able to grow, optimally, in mice receiving the maximum tolerated dose of the respective antifolic compounds. An analysis of the mechanism of development of resistance in leukemic cells is of paramount importance in the development of a rational approach to the problem of chemotherapy of leukemia. Studies are now in progress in this laboratory to investigate the mechanism involved. In the meantime we wish to report the following observations on some properties of the resistant variants: 1) the effects of other known antileukemic agents on one of the resistant variants, the subline resistant to amethopterin, 2) the behavior of resistant leukemic cells carried in serial transplant in the

[1] Received for publication December 11, 1950.

[2] These compounds and other folic acid analogs mentioned in the text were kindly supplied by the Lederle Laboratories, Pearl River, New York.

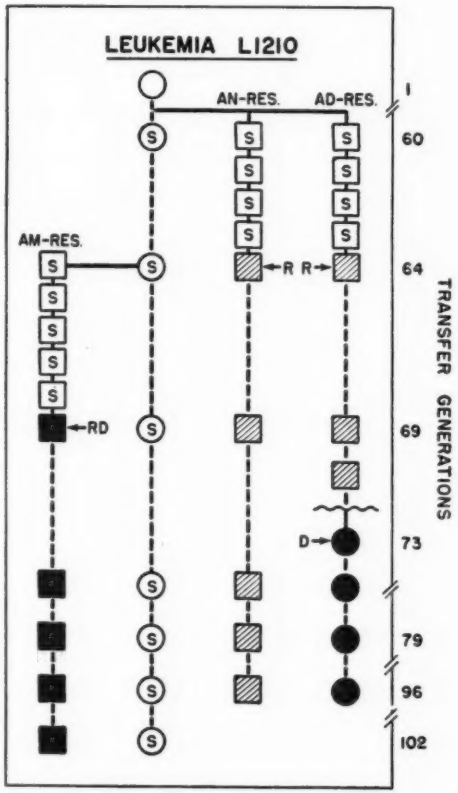
absence of the specific folic acid antagonist—this was investigated in the subline which was found to be resistant to adenopterin—and 3) the development of dependence on folic acid antagonists for optimal and continued growth in two of the sublines under investigation, the sublines resistant to adenopterin and amethopterin.

MATERIALS AND METHODS

Acute lymphoid leukemia, L1210 (7) and 3 resistant sublines which are hereafter designated AM-resistant (resistant to amethopterin), AD-resistant (resistant to adenopterin) and AN-resistant (resistant to aninopterin), have been used in the present study. The morphology and biologic behavior of the susceptible control subline of this transplantable leukemia have been closely observed. The principal cell is large and mononucleated, with a large vesicular nucleus and prominent nucleoli, a distinct nuclear membrane, and a narrow rim of basophilic cytoplasm. There is little variation in the size or morphology of the cells, and mitotic figures are frequent.[3] Numerous cells are dispersed in lymph nodes, spleen, and liver, enlarging these organs and obscuring the architecture. Infiltration is severe following transfer of cells intraperitoneally. The leukemic cells appear in the peripheral blood a few days after transplantation; very high leukocyte counts develop in mice inoculated intraperitoneally, whereas a moderate leukocytosis appears following transplantation by the subcutaneous route. These lines have been carried in transplant in strain dba, subline 212, mice by inoculations subcutaneously of a Locke's-solution suspension of leukemic cells. An attempt has been made to control adequately the number of leukemic cells inoculated at each transfer by using a 20 percent suspension by weight of lymphomatous tumor tissue and inoculating 0.1 cc. (approximately 800,000 leukemic cells) into the right axillary region.

Sublines AD-resistant and AN-resistant leukemic cells were developed from the 60th transfer of the susceptible, control leukemia, L1210, by consecutive transfers, subcutaneously, through mice receiving the approximate maximum tolerated dose of these compounds, 3 mg. per kilogram of body weight for 10 days. Transfers were always accomplished as soon as possible when the localized growth of leukemic cells was large enough to transfer in a brei suspension. Subline AM-resistant was developed in like manner from the 65th transfer of the control leukemia. The AM-resistant subline has now been carried through 38 consecutive transfers (32 since emergence of resistance) and the AN-resistant and AD-resistant sublines have been carried through 40 consecutive transfers (35 since emergence of resistance). Injection of amethopterin and aninopterin, respectively, has been continued in host animals bearing transplants of the AM-resistant and AN-resistant leukemic cells, while the AD-resistant subline has been carried without adenopterin, except as stated in the text, for 23 consecutive transfers, beginning at the 73d transfer, 9 transfer generations after emergence of resistance (text-fig. 1).

[3] Personal communication from Dr. Thelma B. Dunn, Pathology Section, National Cancer Institute.



TEXT-FIGURE 1.—Showing origin of aninopterin-resistant, adenopterin-resistant and amethopterin-resistant variants of leukemia L1210. S represents cells which are susceptible to folic acid antagonists; R represents resistant cells and D represents dependency on folic acid antagonists for optimal growth. Squares represent serial transfer of leukemic cells in host animals receiving antagonists; circles represent transfer of leukemic cells in antagonist-free hosts. Cross-hatching represents resistant cells; block figures represent leukemic cells that are both resistant and dependent. Waved line at transfer generation 73 in AD-resistant subline represents subsequent transfer of the resistant and dependent variant in antagonist-free hosts. All transfers of leukemic cells accomplished at 9 days except as noted in text.

Strain dba, subline 212, mice of both sexes, weighing from 16 to 20 grams were used as host animals for the transfer of leukemic cells. They were kept in an air-conditioned room in plastic cages, not more than 7 to a cage, and were given an unlimited supply of Derwood Mills' cubes and tap water.

We have used throughout the experiments reported here, as a measure of the growth of leukemic cells, the mean weight of the localized lymphomatous mass at 9 days following subcutaneous transfer of leukemic cells. A discrete, localized mass of lymphomatous tissue is found follow-

ing inoculation subcutaneously. This growth is first palpable in the control and resistant sublines at 72 to 96 hours. The mass of tissue is easily removed by forceps and weighed to the nearest milligram on a Roller-Smith torsion balance.

RESULTS

Folic acid antagonists have been found in this laboratory to be the most efficacious of all reported antileukemic agents in increasing the life span of mice with acute lymphoid leukemia. Table 1 is a summary of a sample group of experiments of the effect on survival time of the 3 antifolic compounds used in this study: amethopterin (*8*), aninopterin, and adenopterin (*9*). Amethopterin has been found to be effective in increasing survival time to nearly 100 percent at a dosage level as low as 0.5 mg. per kilogram, given every other day for 10 days. It can be shown, under appropriate experimental conditions, that a true quantitative antagonism exists between the folic acid antagonist used and the parent compound. For example, at a ratio of folic acid to amethopterin of 10:1, complete reversal of the antileukemic effect is obtained providing folic acid is administered 1 or 2 hours prior to the antagonist. Furthermore, it can be shown that on a fixed dose of folic acid, the degree of inhibition of leukemic cell growth, as measured by survival time, varies with the dose of antagonist given.[4]

TABLE 1.—*Effect of folic acid antagonists on survival time of strain dba mice bearing transplantable acute lymphoid leukemia, L1210*

Compound	Number of experiments	Number of mice [1]	Dosage (mg./K)		Survival time days (range)	Percent increase survival time
			Daily [2]	Total		
Amethopterin	4	42	3	24–27	20. 7±0. 61 (11–35)	127. 4
Adenopterin	4	36	3	24–27	18. 9± . 36 (15–27)	107. 7
Aninopterin	4	39	3	24–27	17. 8± . 37 (13–25)	95. 6
Control	4	41	None		9. 1± . 59 (7–13)	------

[1] Strain dba mice of both sexes weighing 16–20 grams were used. Approximately 800,000 leukemic cells in Locke's solution were inoculated intraperitoneally.

[2] Compounds were given intraperitoneally every other day beginning at 48 hours postinoculation of leukemic cells. Control mice gained 1.8 grams and experimental mice maintained their weight at 10 days.

EFFECT OF OTHER ANTILEUKEMIC AGENTS ON LEUKEMIC CELLS OF THE AM-RESISTANT SUBLINE

The possibility was considered that the resistance developed to a folic acid antagonist might extend to other agents exerting specific antileukemic effects. The effect of such agents was studied on a selected resistant subline, the AM-resistant line. 8-Azaguanine (guanazolo) (*10, 11*), alpha-peltatin (*12*), 2,6-diaminopurine (*13*) and Compound E, cortisone [5] (*14*), all of which have been reported to show leukemia-inhibiting properties, were used at the approximate maximum tolerated doses shown in table 2.

[4] Unpublished observations.

[5] 8-Azaguanine (5-amino-7-hydroxy-1-v-triazolo [d] pyrimidine) was kindly supplied by the Lederle Laboratories, Pearl River, N. Y. Alpha-peltatin was supplied by Dr. Joseph Leiter, Chemotherapy Section, National Cancer Institute.

2,6-diaminopurine lactate was supplied by Dr. Joseph Burchenal, Sloan-Kettering Institute, New York.

TABLE 2.—*Comparative sensitivity of resistant (AM-resistant) and susceptible leukemic cells to several antileukemic agents*

Experiment	Susceptible L1210					Experiment	AM Resistant L1210				
	Number mice	Dosage (mg./k)		Mean lymphoma weight (mg.)	Inhibition index		Number mice	Dosage (mg./k)		Mean lymphoma weight (mg.)	Inhibition index
		Daily	Total					Daily	Total		
8-Azaguanine	55	37. 5–50	300–400	93. 2	0. 13	8-Azaguanine	67	37. 5–50	300–400	43. 5	0. 17
Control	48	None		735. 5		Control	49	None		407. 8	
Alpha-peltatin	19	3. 75–5. 0	11. 3–15. 0	71. 8	. 06	Alpha-peltatin	29	3. 75–5. 0	11. 3–15. 0	30. 8	. 06
Control	15	None		1, 119. 5		Control	15	None		489. 9	
Aninopterin	18	3	12	32. 5	. 04	Aninopterin	12	3	12	393. 6	1. 03
Adenopterin	24	3	12	6. 5	. 01	Adenopterin	12	3	12	293. 5	. 78
Control	24	None		764. 0		Control	12	None		383. 3	
Folic acid [1]	15	30	120	524. 0	------	Folic acid [1]	34	30	120	159. 0	------
Control	15	None		589. 6	------	Control	20	None		207. 7	------
						Folic acid [2]	6	30	120	634. 3	------
						Control	6	None		491. 6	------

Transfer generations 77–87 of AM-Resistant Leukemia L 1210 used except: [1] Where transfers 72-76 and [2] transfer 79 were used.

8-Azaguanine was dissolved in 0.01 N NaOH and injected subcutaneously daily beginning at 48 hours following inoculation of leukemic cells. A 0.133-percent fresh solution was used for injections and 0.05 cc. was injected per gram of body weight. Alpha-peltatin was dissolved in 1 N NaOH and diluted with Locke's solution to a volume permitting injection of 0.01 cc. per gram of body weight. Injections were done subcutaneously on the left sides on the 3d, 5th, and 7th days following inoculation of leukemic cells. 2,6-Diaminopurine in the form of the lactate containing 44 mg. per cc. of solution was used at various dosage levels: 74, 83, 88, 92, and 110 mg. per kilogram of body weight and injected either subcutaneously or intraperitoneally every other day for a total of 4 injections. Dilutions in Locke's solution were adjusted so that 0.005 cc. per gram of body weight was injected at each dosage level. Cortisone acetate (Compound E) in aqueous suspension was injected intraperitoneally at a dosage level of 50 mg. per kilogram of body weight. Injections were commenced 48 hours postinoculation, and continued for 7 successive days. Dilutions were adjusted to allow for the injection of 0.005 cc. per gram of body weight. The folic acid antagonists dissolved in distilled H_2O were injected intraperitoneally beginning at 48 hours after inoculation of leukemic cells. Dilutions were made so that 0.01 cc. was injected per gram of body weight.

Transfer generations 77 through 87 of the AM-resistant sublines were used to test the effect of these antileukemic agents and corresponding transfer generations of the susceptible L1210 subline were used as control material.

Cortisone and 2,6-diaminopurine did not produce a significant inhibition of the growth of leukemic cells either in the control, susceptible subline, or in the AM-resistant subline at the dosage levels used, and are not included in the tabular material. It can be seen by reference to table 2 that 8-Azaguanine strikingly inhibited the growth of lymphomatous cells in both the susceptible subline and in the subline resistant to amethopterin. The inhibition index, that is, the ratio of the mean weight of lymphomatous tissue in the treated group to the mean weight of lymphomatous tissue in the control group, is practically identical in the two groups. Similarly, the degree of inhibition of growth of lymphomatous cells by alpha-peltatin in the susceptible and AM-resistant sublines is identical. In contrast to the observed effects of unrelated antileukemic agents on the growth of susceptible and resistant leukemic cells, an unequivocal, cross-resistance was obtained when AM-resistant leukemic cells were grown in mice receiving two closely related antifolic compounds, adenopterin and aninopterin. Nearly complete inhibition of growth at 9 days was observed in the susceptible subline, and optimal growth was obtained in the AM-resistant subline. This characteristic cross-resistance was also observed in another resistant subline, AD-resistant. These resistant leukemic cells showed cross-resistance to 4-amino-pteroylglutamic acid (aminopterin) and 4-amino-pteroylaspartic acid (amino-an-fol) as well as to the two antifolic compounds reported here.

No discernible effect of the parent compound, pteroylglutamic (folic) acid, was observed in either the resistant or susceptible subline (table 2).

The Behavior of Resistant Leukemic Cells Carried in Serial Transfer Without Folic Acid Antagonist

The resistant subline of leukemic cells, AD-resistant, has now been carried through 23 successive transfers, except as noted below, in host animals that were not given the specific folic acid antagonist, adenopterin, which was used in developing the resistance. At the 88th transfer, 15 transfer generations following establishment of this subline in antagonist-free animals, a diminished growth rate of leukemic cells was evident. These animals were then given 2 injections of adenopterin at a dosage level of 3 milligrams per kilogram of body weight, at 8 and 10 days postinoculation of leukemic cells. There resulted a profuse growth of cells that were transferred again into antagonist-free animals at 13 days. In all other transfer generations the resistant leukemic cells were inoculated routinely at 9 days postinoculation. No change has been observed in the susceptibility of these cells to adenopterin during this period of transfer through antagonist-free hosts. Resistance to this specific folic acid antagonist may, therefore, be considered a stable and irreversible heritable change. Table 3 presents data on the emergence of resistance in this subline and sample results at various transfer periods.

TABLE 3.—*Data showing origin of resistance, stability of the resistant variant and partial dependency upon adenopterin in the AD-resistant subline*

Transfer generation	Number mice	Dosage (mg./k)		Lymphoma weight (mg.) at 9 days	Dependency ratio	Remarks
		Daily	Total			
60–64	25	3	20–24	None		
65	7	3	12	104. 9		Emergence of resistance
66–76	50	3	12	446. 5		
73	10			409. 9		Serial transfer without adenopterin.
75	5	3	12	334. 2	2. 3	
	5			148. 9		
79	5			542. 9		
81	5	3	12	1, 271. 0	4. 2	
	5			300. 0		
84	3			475. 0		
88	5	3	12	116. 0	3. 2	
	5			35. 7		
95	5	3	12	713. 0	2. 8	
	5			264. 3		
96	5	3	12	732. 3	4. 5	
	5			160. 9		

It is to be noted, 1) that resistance to adenopterin, at a dosage level of 3 mg. per kilogram of body weight given every other day for 4 days, has persisted through 23 transfer generations in antagonist-free hosts, and 2) that specific enhancement of growth results in animals receiving the antagonist. This indicates a partial dependency of the resistant leukemic cells of this subline upon the antagonist for growth.

929548—51——22

It is to be noted also that considerable variation in the mean weights of the subcutaneous lymphomatous tissue was found among the different transfer generations grown in mice not given the antagonist. In contrast, relatively little variation in mean weights of the subcutaneous lymphomatous tissue was found in the AM-resistant subline carried in serial transfer in hosts receiving a standard dose of antagonist (table 4).

TABLE 4.—*Dependence of resistant leukemic cells (AM-resistant subline) on amethopterin for optimal growth*

Experiment No.	Transfer generation	Number mice	Dosage mg./k Daily	Dosage mg./k Total	Mean lymphoma weight in milligrams	Dependency ratio
1	77	12	3	12	750.2 (322.0–981.0)	2.2
		13	None		336.5 (228.1–508.2)	
2	79	7	3	12	365.7 (211.1–453.3)	2.5
		6	None		146.5 (75.7 –254.9)	
3	80	5	3	12	454.8 (246.8–818.0)	2.7
		5	None		172.8 (135.6–211.0)	
4	81	5	3	12	312.1 (213.5–365.0)	1.1
		5	None		274.2 (140.1–297.7)	
5	82	5	3	12	392.5 (176.8–680.0)	3.4
		5	None		116.3 (88.5–160.0)	
6	83	7	3	12	413.1 (240.2–700.0)	2.9
		6	None		145.3 (46.0–277.3)	
7	84	5	3	12	579.5 (410.8–690.0)	2.9
		5	None		151.7 (95.0–240.0)	
8	85	5	3	12	457.3 (259.8–550.0)	2.3
		5	None		198.4 (165.9–235.7)	
9	86	5	3	12	432.9 (280.6–552.6)	1.8
		5	None		235.6 (88.0–501.4)	
10	87	5	3	12	343.6 (255.3–429.7)	6.8
		5	None		50.1 (36.2– 60.5)	
Total		61	3	12	480.7±26.5	2.4
		60	None		202.1±17.9	
	Difference of means=278.6±31.9 mg; P<0.001					

DEPENDENCE OF RESISTANT LEUKEMIC CELLS ON FOLIC ACID ANTAGONIST FOR OPTIMAL GROWTH

We have presented previously (*6*) preliminary information indicating a partial dependence of resistant leukemic cells on folic acid antagonists for optimal growth. A more extensive analysis of the dependency characteristic of one of the resistant variants, AM-resistant, is given in tables 4 and 5. In a sample of 10 transfers, selected between the 77th and 87th transfer generations of the AM-resistant subline, it can be seen that striking increases in mean lymphomatous tumor weights were obtained when the host animals were supplied with amethopterin in contrast to the growth of these leukemic cells in mice deprived of the antagonist. On the other hand, the concentration of antagonist used, 3 mg. per kilogram of body weight given for 4 days beginning 24 hours postinoculation of leukemic cells, apparently interferes with an essential metabolic process in the susceptible control leukemic cells, resulting in nearly complete inhibition of growth.

TABLE 5.—*Comparative sensitivity of resistant (AM-resistant subline) and susceptible (Control subline) leukemic cells to amethopterin*

Leukemic cells	Transfer generations	Number mice	Dosage (mg./k)		Mean lymphoma weight (mg.)
			Daily	Total	
1. Resistant (AM-resistant subline).	77–87	61	3	12	480.7±26.5
		60	None		202.1±17.9
2. Susceptible (control subline)	65–69	25	3	12	No growth
	70	5	None		494.3
	73–85	43	3	12	10.3 [1]
	73–85	47	None		732.2±41.4

[1] Only 9 growths appeared in this series ranging from 10.7 to 28.6 milligrams, mean weights of experimental groups.

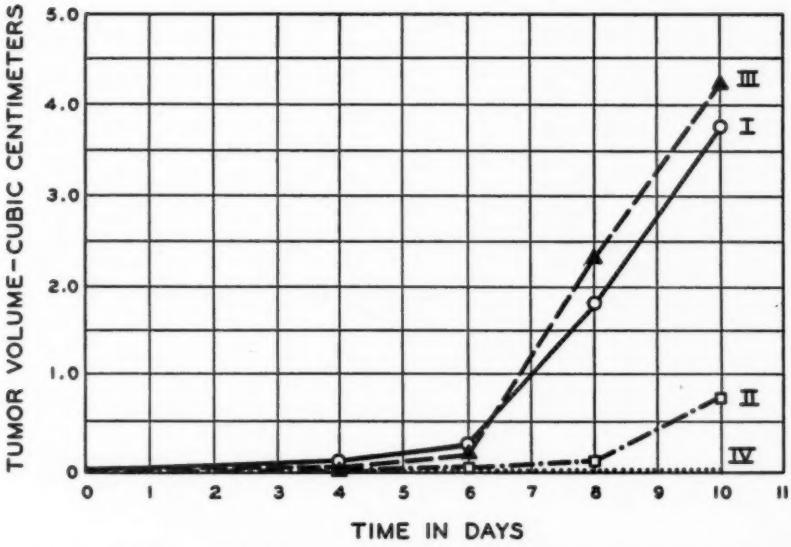
It has been observed that the antagonist-resistant sublines of leukemia, when grown in mice supplied with the respective folic acid antagonists, resemble in biologic behavior the susceptible control subline. In addition to a profuse localized growth of the lymphomatous cells, there occurs an extensive infiltration of cells into the lymph nodes, spleen, and liver, greatly enlarging these organs and obscuring the architecture. Figures 1 and 2 show characteristic localized growth of the resistant leukemic cells. Escape of leukemic cells into the peripheral blood and a leukocytosis closely resembling the blood picture of mice bearing transplants of the control subline are obtained. A distinct leukemic blood picture is found in mice bearing resistant cells even though the dosage of antagonist is increased to a level of 5 mg. per kilogram of body weight. Table 6 gives a general comparison of the peripheral blood picture and of organ infiltrations in the 101st transfer generation of the AM-resistant subline and the corresponding transfer generation of the susceptible, control subline. The mean survival time of strain dba mice bearing subcutaneous transplants of resistant leukemic cells, and given the antagonist parenterally, is strikingly similar to the mean survival time of dba mice bearing transplants of the susceptible subline. For example, the mean survival time of a sample of 15 dba mice bearing AM-resistant cells and given 3 mg. of amethopterin per kilogram of body weight every other day for 4 days was 12.4 ± 0.33 days, and the mean survival time of 15 dba mice bearing a corresponding transfer of the susceptible, control leukemic cells was 12.7 ± 0.15 days.

The growth rates of the AM-resistant variant cells and of the control susceptible leukemic cells following transplantation of the 102d transfer generation of each subline are compared in text-figure 2. The growth rates of susceptible leukemic cells grown in untreated dba mice (Curve I) and AM-resistant cells grown in mice given the maximum tolerated dose of amethopterin (Curve III) are similar. On the other hand, the mean tumor volume attained at 10 days of AM-resistant leukemic cells grown in antagonist-free mice was only 0.84 cc. (Curve II) compared with a mean tumor volume of 4.26 cc. when grown in mice given amethopterin. Com-

TABLE 6.—*Peripheral blood counts and organ infiltrations at 9 days in strain dba mice bearing subcutaneous transplants of Amethopterin-resistant and susceptible lymphoid leukemia L1210*

Transfer line	Dosage (mg./k)		Hemoglobin gm. percent	Peripheral blood counts				Organ infiltrations [4]		
	Daily [1]	Total		wbc	Agranulocytes	Granulocytes	Lymphoblasts	Lymph nodes	Spleen	Liver
AM-resistant [2 3]	3	12	10. 1	47, 760	11, 940	32, 299	3, 821	+++	+++	++
	5	20	12. 0	24, 930	7, 719	16, 000	1. 211	+++	++	+
Susceptible control	None		11. 2	41, 150	14, 568	20, 821	5, 761	+++	+++	++
	3	12	13. 5	11, 025	6, 550	4, 520	55	−	−	−

[1] Injections of amethopterin given intraperitoneally every other day beginning at 48 hours following inoculation of leukemic cells.
[2] 5 animals in each group used to determine mean figures for blood counts.
[3] Mean weight of lymphoma tissue as determined on 10 mice in each group was as follows: group 1, 1,362.7 milligrams; group 2, 1,146.0 milligrams; group 3, 1,478.0 milligrams; and group 4, no growth.
[4] +++ severe infiltration of leukemic cells greatly enlarging organs and obstructing architecture, ++ moderate infiltration, + slight infiltration, − no infiltration, as determined by microscopic study.



TEXT-FIGURE 2.—Tumor volume measurements of amethopterin-resistant leukemic cells of the 102d transfer grown in strain dba mice receiving amethopterin (Curve III) and in antagonist-free mice (Curve II). Tumor volume measurements of the 102d transfer of susceptible leukemic cells are shown in Curve IV, grown in mice treated with amethopterin at a dosage level of 3 milligrams per kilogram for 4 days and in Curve I grown in untreated mice. Each point on curves represents the mean measurement of 7 mice.

plete inhibition of the growth of leukemic cells of the susceptible subline grown in mice receiving the maximum tolerated dose of amethopterin was observed in this transfer (Curve IV).

No changes in morphology, antigenicity, or transplantability have been observed between the susceptible and resistant cells of this leukemia. Leukemic cells of the susceptible or resistant sublines as observed in localized growth or in infiltrations into spleen, liver, and lymph nodes are morphologically indistinguishable. Attempts to detect antigenic differences by complement-fixation tests have been unsuccessful.[6] Leukemic cells of the susceptible, control subline grow progressively, causing death of the host only in strain dba, subline 212, mice and in F_1 hybrids using strain dba as one of the parents. No changes in the transplantability of cells of the resistant sublines have been observed. These cells have failed to grow in mice of the C57 black, C58, B alb C, RIL, and C3H strains.

DISCUSSION

Evidence has been presented that shows the development of resistance in leukemic cells of the mouse, lymphoid in type, to each of 3 folic acid antagonists. In 2 of the transplant sublines developed, the AM-resistant

[6] These tests were done by Dr. Richard Malmgren of this Institute.

and AD-resistant sublines, it is apparent that the resistant leukemic cells are dependent on the presence of the antagonist for optimal growth. A relatively restricted growth of resistant cells is obtained in mice deprived of antagonist so that this must be considered as a partial dependency. The dose of antagonist that interferes with growth of the control-susceptible cells appears to be essential for optimal growth of resistant cells. The third subline of resistant cells developed, AN-resistant, continues to be resistant through serial transplantation in hosts given the folic acid antagonist, aninopterin, at the maximum tolerated dosage level, but it has not been tested adequately for dependency.

The transformation that has occurred in these leukemic cells appears to be a stable and irreversible heritable change as determined by growing one of the resistant sublines, AD-resistant, in serial transfer in hosts not given the folic acid antagonist. During this period the variant cells have remained resistant to and partially dependent on the specific folic acid antagonist used in developing resistance.

These transformations appear to be similar to the changes occurring in bacteria that are grown in the presence of antibiotics. Numerous investigators, using the method devised by Luria and Delbrück (*1*), for determining the manner of origin of resistant bacteria, have concluded that resistance originates through genetic changes, and that the agent used acts only in a selective manner, eliminating susceptible bacteria and allowing the majority of resistant forms that are always present in a large population of bacteria to reproduce.

Experiments now in progress indicate that the changes that occur in this particular leukemia under investigation are discrete, step-wise, changes. When leukemic cells, for example, are transplanted serially to groups of mice given different concentrations of amethopterin, from 0.5 to 4.0 mg. per kilogram of body weight, the data indicate increases in resistance at different magnitudes occurring in a random manner and separated during several transfers by definite plateaus. Thus, it would appear that the pattern of resistance obtained here, to folic acid antagonists, is similar to the pattern of resistance of several types of bacteria to penicillin (*3*, *5*). The method used in this study to obtain the original resistant variants precluded an exact determination of the pattern of resistance.

Data on the crucial test to determine the manner of origin of resistant leukemic cells are not yet available. This consists of comparing the variation in response to an antileukemic agent in a series of independent transfers of leukemic cells with variation of response in a series derived from a single transfer. By this method it should be possible to determine the manner of origin of the variants under consideration, that is whether they are induced by the compound in use or whether the compound acts in selecting spontaneously occurring mutations.

It is indicated that there is a difference in the mechanism of effect of 8-Azaguanine and alpha-peltatin from the folic acid antagonists since cross-resistance was not demonstrated with these compounds. Since

all of the folic acid antagonists used in this study showed characteristic cross-resistance, it appears that they inhibit leukemic-cell growth in an identical or similar manner.

If mutation and selection prove to be the mechanism involved in the origin of resistance to various antileukemic agents, the situation as to the use of various agents in the treatment of leukemia would be well defined. It would consist of the earliest possible treatment at maximum tolerated dosages continued as long as possible employing proved antileukemic agents that are known to be independent of each other in their actions. The test of independence, as shown above, is that leukemic cells that have developed a resistance to one antileukemic agent are still susceptible to other agents. Only leukemic cells that are resistant to the mixture of antileukemic agents should develop as resistant forms. Theoretically, however, these resistant variants should be relatively rare. Since highly resistant variants have been found even when using the most effective antileukemic agents, the folic acid antagonists, it is to be expected that all of the leukemic cells will not be eliminated. In the case of bacteria, by use of high concentrations of antibacterial agents, it is possible to reduce the number of susceptible bacteria to a level with which the organism is capable of dealing, resulting eventually in elimination of bacteria. In the case of leukemic cells, however, this situation probably may never be attained, since it has been shown by Furth and Kahn (*15*) that even a single leukemic cell introduced into a host animal will develop into a generalized leukemia within a relatively short period of time.

Combination therapy studies now in progress, using amethopterin, 8-Azaguanine and alpha-peltatin at dosage levels that do not appreciably influence the general health of the animal, indicate a striking effect in inhibition of leukemic-cell growth and survival time on the acute lymphoid leukemia, L1210, used in this study. These results will be published later.

SUMMARY

A transplantable acute lymphoid leukemia has given rise to 3 variants when grown in serial transfer in mice receiving the maximum tolerated dose of 3 different folic acid antagonists: amethopterin, adenopterin and aninopterin.

In the case of one of these, the adenopterin-resistant variant, which was tested, susceptibility to the specific antagonist has remained unchanged following 23 consecutive serial transfers in mice free of the antagonist. This is evidence of a stable and irreversible heritable change.

Concentrations of folic acid antagonists that nearly completely inhibit growth of lymphomatous tissue of the susceptible subline appear to be essential for optimal growth of 2 of the variants, the sublines resistant to amethopterin and adenopterin, respectively. The 3d variant described in this study has not been tested adequately relative to dependence.

The amethopterin-resistant variant has been shown to be cross-resistant to all other folic acid antagonists tested but sensitive to two other antileukemic agents, 8-Azaguanine and alpha-peltatin. Cortisone and 2,

6-diaminopurine failed to inhibit growth of leukemic cells of either the susceptible or resistant sublines.

The biologic behavior of resistant leukemic cells, grown in mice supplied with folic acid antagonist, appears to be identical to the behavior of susceptible cells grown in untreated mice.

There have been observed no changes in morphology, antigenicity or transplantability in the transformed leukemic cells.

Considerations of the manner of origin of these changes and the significance of these findings to the chemotherapy of leukemia are presented.

REFERENCES

(*1*) Luria, S. E., and Delbruck, M.: Mutations in bacteria from virus sensitivity to virus resistance. Genetics 28: 491–511 (1943).

(*2*) Demerec, M.: Production of staphylococcus strains resistant to various concentrations of penicillin. Proc. Nat. Acad. Sc. 31: 16–24 (1945).

(*3*) ———: Origin of bacterial resistance to antibiotics. J. Bacteriology 56: 63–74 (1948).

(*4*) Witkin, E. M.: Genetics of resistance to radiation in *Escherichia coli*. Genetics 32: 221–248 (1947).

(*5*) Oakberg, E. F., and Luria, S. E.: Mutations to sulfonamide resistance in *Staphylococcus aureus*. Genetics 32: 249–261 (1947).

(*6*) Law, L. W., and Boyle, P. J.: Development of resistance to folic acid antagonists in a transplantable lymphoid leukemia. Proc. Soc. Exper. Biol. & Med. 74: 599–602 (1950).

(*7*) Law, L. W., Dunn, T. B., Boyle, P. J., and Miller, J. H.: Observations on the effect of a folic-acid antagonist on transplantable lymphoid leukemias in mice. J. Nat. Cancer Inst. 10: 179–192 (1949).

(*8*) Smith, J. M., Jr., Cosulich, D. B., Hultquist, M. E., and Seeger, D. R.: The chemistry of certain pteroylglutamic acid antagonists. Tr. N. Y. Acad. Sc. 10: 82–83 (1948).

(*9*) Hultquist, M. E., Smith, J. M., Jr., Seeger, D. R., Cosulich, D. B., and Kuh, E.: Analogs of pteroylglutamic acid II. 9-methylpteroylglutamic acid and derivatives. J. Am. Chem. Soc. 71: 619–622 (1949).

(*10*) Kidder, G. W., Dewey, V. C., Parks, R. S., Jr., and Woodside, G. L.: Purine metabolism in Tetrahymena and its relation to malignant cells in mice. Science 109: 511–514 (1949).

(*11*) Law, L. W.: Studies on the effects of a guanine analog on acute lymphoid leukemias of mice. Cancer Research 10: 186–190 (1950).

(*12*) Greenspan, E. M., Leiter, J., and Shear, M. J.: Effect of alpha-peltatin, beta-peltatin and podophyllotoxin on lymphomas and other transplanted tumors. J. Nat. Cancer Inst. 10: 1295–1333 (1950).

(*13*) Burchenal, J. H., Bendich, A., Brown, G. B., Eleon, G. V., Hitchings, G. H., Rhoads, C. P., and Stock, C. C.: Preliminary studies on the effect of 2, 6-diaminopurine on transplanted mouse leukemia. Cancer 2: 119–120 (1949).

(*14*) Heilman, F. R., and Kendall, E. C.: The influence of 11-dehydro-17-hydroxycorticosterone (Compound E) on the growth of a malignant tumor in the mouse. Endocrinology 34: 416–420 (1944).

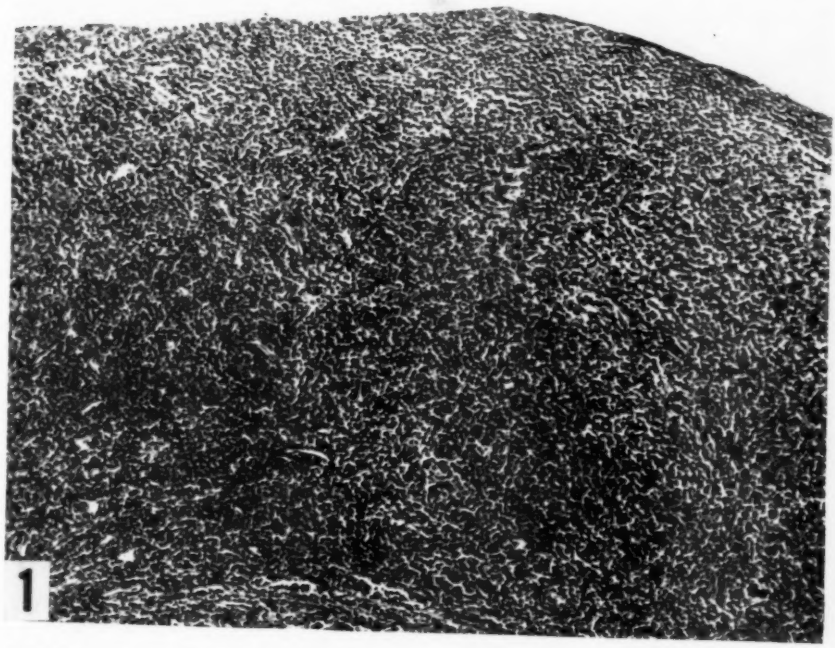
(*15*) Furth, J., and Kahn, M. C.: The transmission of leukemia of mice with a single cell. Am. J. Cancer 31: 276–291 (1937).

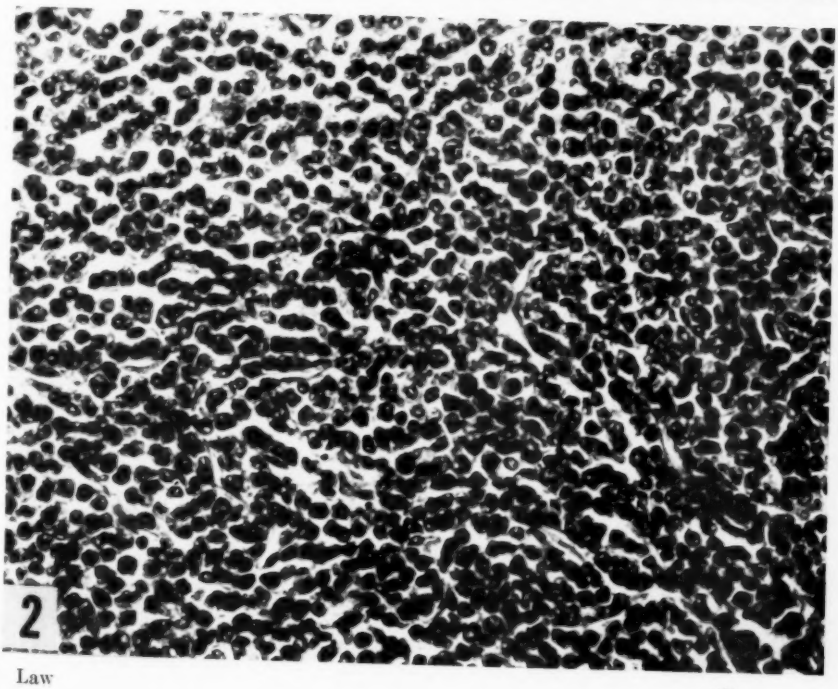
PLATE

929548—51——23

PLATE 90

FIGURES 1 and 2.—Subcutaneous implant of lymphomatous tissue of AM-resistant subline grown in a strain dba mouse receiving 3 milligrams per kilogram of body weight of the folic acid antagonist, amethopterin. This was the 94th transfer generation. Magnification for figure 1, × 112; for figure 2, × 415.





Law

U. S. GOVERNMENT PRINTING OFFICE: 1951